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CAROLINE MARIA CHRISTANTE

ESTUDOS EXPERIMENTAIS DO EFEITO DO DI-N-BUTIL FTALATO E DO MONO-(2-ETILHEXIL) FTALATO SOBRE O DESENVOLVIMENTO TESTICULAR DE ROEDORES

EXPERIMENTAL STUDIES OF THE DI-N-BUTYL PHTHALATE AND MONO-(2-ETHYLHEXYL) PHTHALATE EFFECT ON TESTICULAR DEVELOPMENT IN RODENTS

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutora em Biologia Celular e Estrutural, na área de Biologia Celular.

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“RENDA-SE, COMO EU ME RENDI. MERGULHE NO QUE VOCÊ NÃO CONHECE COMO EU MERGULHEI. NÃO SE PREOCUPE EM ENTENDER, VIVER ULTRAPASSA QUALQUER ENTENDIMENTO.”

(Clarice Lispector)

RESUMO

Os ésteres de ftalatos tais como o di-n-butil ftalato (DBP), o di-(2-etilhexil) ftalato (DEHP) e o seu metabolito ativo, mono-(2-etilhexil) ftalato (MEHP), podem exercer efeitos adversos nos hormônios reprodutivos e nas células germinativas fetais e neonatais de maneira espécie-específica. Por outro lado, o ácido docosahexaenoico (DHA - 22:6 n-3), um ácido graxo polinsaturado de cadeia longa (PUFA) – abundantemente encontrado em fontes oleosas – tem se mostrado benéfico para a produção de testosterona, para a espermatogênese e para a motilidade espermática. Sabe-se, também, que o MEHP e o DHA atuam nas mesmas vias metabólicas envolvidas na síntese de colesterol/lipídeos, mas de maneiras antagônicas. O presente estudo avaliou a exposição materna ao DBP sob o desenvolvimento do testículo do gerbilo da Mongólia, nas seis primeiras semanas do desenvolvimento pós-natal. Além disso, avaliou os efeitos *in vitro* do DHA sozinho ou em combinação com o MEHP sob o testículo fetal de camundongo, utilizando o sistema de cultura organotípica. Para tanto, os testículos de gerbilos machos nascidos de mães controle (C) ou expostas, durante a gestação, ao óleo mineral (O) ou ao DBP (100 mg/kg, do dia 8 ao dia 23 pós-concepção) foram avaliados nas idades de 1, 7, 14, 28, 35 e 42 dias. Também foram realizadas análises *in vitro* de testículos de camundongos de 13,5 dias pós-concepção cultivados apenas em meio de cultura (controle), seja com DHA (50μM), seja com MEHP (20μM) ou com ambos (50μM de DHA e 20μM MEHP), após 24 ou 72 horas. Nossos resultados indicaram que o DBP comprometeu a densidade de figuras mitóticas no nascimento e o número total de gonócitos (NG) aos 7 dias, além de aumentar a atividade esteroidogênica no final da primeira semana de vida do gerbilo. O óleo mineral, por sua vez, diminuiu os níveis plasmáticos de testosterona e elevou os de estrógeno nos 7^{o} e 28^{o} dias, respectivamente. As análises *in vitro* indicaram que o MEHP não alterou o número de gonócitos. No entanto, o DHA estimulou a secreção de testosterona após 72h. Esse lipídeo também induziu o extravasamento marcante de gonócitos para o tecido intersticial, um efeito pró-apoptótico das populações de células no estroma da gônada e alterações morfológicas nos cordões testiculares, após 3 dias de cultura. Sendo assim, os experimentos *in vivo* demonstraram que a primeira semana de vida pós-natal do gerbilo é mais sensível aos efeitos deletérios do ftalato, no tocante ao número de células germinativas e à esteroidogênese. As análises *in vitro*, por conseguinte, indicaram que o desenvolvimento testicular foi prejudicado pela exposição ao DHA, a qual induziu alterações degenerativas nas células de Sertoli, intensificadas quando em combinação com o MEHP.

ABSTRACT

Phthalate esters such as di-n-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP) and its active metabolite, mono-(2-ethylhexyl) phthalate (MEHP), may exert adverse effects on reproductive hormones and on fetal/neonatal germ cells in a species-specific manner. On the other hand, the docosahexaenoic acid (DHA - 22:6 n-3), a long-chain polyunsaturated fatty acid (PUFA) abundantly found in oily fish sources, seems to be beneficial for testosterone production, spermatogenesis and sperm motility. It is also known that MEHP and DHA act on the same metabolic pathways involved in cholesterol/lipid synthesis but in antagonistic ways. Thus, the present study evaluated whether the maternal exposure to DBP interferes with the development of the Mongolian gerbil testis during the first six weeks of postnatal development. It evaluated the action of DHA alone or in combination with MEHP on the mouse fetal testis as well, using the organotypic culture. For this, testis of male gerbils born from control (C) or exposed pregnant mothers to mineral oil (O) or DBP (100 mg/kg from 8 to 23 days post conception) were checked at the ages of 1, 7, 14, 28, 35 and 42 days. It was also conducted *in vitro* analysis of mice testes at 13.5 days post-conception (dpc) cultured in medium only (controls), DHA (50µM), MEHP (20µM) or both (50µM DHA/20µM MEHP) for 24 or 72 hours. As demonstrated by our results, DBP impaired the density of mitotic figures at birth and the total number of gonocytes (NG) at 7 postnatal days, besides increasing steroidogenic activity at the end of the first week of life, in the gerbil. The mineral oil, on the other hand, decreased testosterone and raised estrogen plasmatic levels at 7 and 28 days, respectively. *In vitro* analisys indicated that MEHP did not change the NG. Nevertheless, DHA stimulated testosterone secretion after 72h. This lipid also induced a marked extravasation of gonocytes into the interstitial tissue, a pro-apoptotic effect of cell populations in the stroma of the gonad and morphological alterations in the testicular cords after 3 days of culture. In conclusion, the present indications provided by *in vivo* experiments demonstrated that the first week of the gerbil postnatal life is more sensitive to deleterious effects of phthalate on the number of germ cells and steroidogenesis. *In vitro* experiments, therefore, indicated that the testicular development, for the mouse, was impaired by expose to DHA, which induced degenerative alterations in the Sertoli cells, worsened when combined with MEHP.

LISTA DE ABREVIATURAS E SÍMBOLOS

17S-H(p)DHA: 17S-hidroxiperóxido-DHA

17β-HSD: 17β-hidroxiesteroide desidrogenase

3β-HSD: 3β-hidroxiesteroide desidrogenase

5OH-MEHP: mono-(2-etil-5-hidroxihexil)

5oxo-MEHP: mono-(2-etil-5-oxohexil)

AAL: ácido alfa-linolênico

ALOX15: enzima 15-lipoxigenase

AMH: hormônio anti-Mülleriano

AR: receptor de andrógenos

BBzP: benzil butil ftalato

BrDU: 5-bromo-2'-desoxiuridina

CGPs: células germinativas primordiais

DBP: di-n-butil ftalato

DEHP: di-(2-etilhexil) ftalato

DEP: dietil ftalato

DEs: desreguladores endócrinos

DHA: ácido docosahexaenoico

DHH: desert hedgehog

DHT: dihidrotestosterona

dpc: dias pós-concepção

dpp: dias pós-parto

EPA: ácido eicosapentanoico

ERα: receptor de estrógeno do tipo α

ERβ: receptor de estrógeno do tipo β

Fgf9: fator 9 de crescimento de fibroblasto

FSH: homônio folículo estimulante

GMNs: gonócitos multinucleados

GnRH: hormônio liberador de gonadotrofina

HCG: gonodotrofina coriônica humana

IGF-1: fator de crescimento semelhante à insulina tipo 1

INSL3: peptídeo 3 semelhante à insulina

LH: hormônio luteinizante

LXR: receptor X do fígado

LysoPC: lisofosfatidilcolina

LysoPC-DHA: DHA complexado à lisofosfatidilcolina

MEHP: mono-(2-etilhexil) ftalato

NGFIB: fator de crescimento do nervo do tipo IB

NRs: receptores nucleares

P450c17: 17α-hidroxilase/17, 20 liase

P450scc: enzima de clivagem da cadeia lateral do colesterol

PC-DHA: DHA complexado à fosfatidilcolina

PPAR: receptor de ativação da proliferação dos peroxissomos

PPARγ: receptor de ativação da proliferação dos peroxissomos do tipo γ

PUFA: ácido graxo polinsaturado de cadeia longa

Rspo-1: R-espondina 1

Sf-1: fator esteroidogênico 1

Sox9: HMG-box 9 relacionado a SRY

SREBP: proteína de ligação a elemento regulador de esterol

SREBP-1: proteína de ligação a elemento regulador de esterol 1

Sry: gene testículo determinante

SSCs: células tronco espermatogoniais

Tfm: síndrome de feminilização testicular

TGFβ: fator de transformação do crescimento beta

Wnt4: membro 4 da família Wnt

Wt-1: gene relacionado ao tumor de Wilms 1

SUMÁRIO

I. INTRODUÇÃO

I. 1. O Desenvolvimento Testicular

O sexo genotípico é fixado na fertilização quando um cromossomo Y ou um cromossomo X adicional se une ao cromossomo X, já existente no ovócito (Carlson, 1994). Durante o desenvolvimento embrionário em mamíferos, a gônada aparece pela primeira vez como um pequeno inchaço que cresce ao longo da superfície celômica dos mesonefron (Jost et al., 1973; Capel, 2000), onde se desenvolve a crista genital, histologicamente igual em ambos os sexos e, por este motivo, denominada gônada bipotencial (Fig. 1). Esta é observada em torno da 4ª semana em humanos e no dia 10,5 pc (pós-concepção), no camundongo (Ross, Capel, 2005; Wilhelm, 2007). O epitélio celômico envia colunas epiteliais curtas para o interior da gônada, para a formação dos cordões sexuais primitivos. Ao se diferenciarem, estes se separam do epitélio germinativo por uma camada densa de tecido conjuntivo – a túnica albugínea. As porções externas dos cordões formarão os túbulos seminíferos, enquanto as porções internas, a rede testis, que se unirá aos ductos eferentes (Carlson, 1994).

O desenvolvimento fenotípico do sexo masculino está relacionado a substâncias secretadas pela gônada em formação. O hormônio anti-Mülleriano (AMH), uma glicoproteína da família TGFβ (fator de transformação do crescimento beta), secretado pelas células de Sertoli, atuará na regressão dos ductos paramesonéfricos. Em contrapartida, a testosterona secretada pelas células de Leydig propiciará o desenvolvimento dos ductos mesonéfricos, que se diferenciarão nos ductos deferentes, nas vesículas seminais e nos epidídimos (Magre, Jost, 1991; Carlson, 1994). As sínteses de testosterona e de AMH dependem da expressão de alguns genes envolvidos na diferenciação dos testículos, tais como: Sf-1 (fator esteroidogênico 1), Wt1 (gene supressor de tumor de Wilms) e Sox9 (HMG-box 9 relacionado a SRY) (Ikeda et al., 1993; Foster et al., 1994; Hastie, 1994; Wagner et al., 1994). A diferenciação testicular inicia-se com a expressão do gene testículo determinante (Sry), localizado no braço curto do cromossomo Y. Na gônada de camundongo, o gene Sry é expresso entre os dias 10,5 e 12,5 pc, unicamente pelas células de Sertoli (Albrecht, Eicher, 2001). Esse tipo celular coordena quase todos os aspectos da morfogênese testicular, podendo surgir de duas fontes: (1) de células SF1-positivas, presentes na gônada bipotencial, ou (2) de células progenitoras, presentes no epitélio celômico. Por volta do dia 10,5 pc, a subpopulação de células SF1-positivas passa a expressar Sry e torna-se comprometida com a linhagem de células de Sertoli (Gubbay et al., 1990; Koopman et al., 1990; Lovell-Badge, Robertson, 1990; Hacker et al., 1995). As células do epitélio celômico que atingem o interior das gônadas

XY dão origem tanto às células de Sertoli como às células intersticiais do testículo (Karl, Capel, 1998; Schmahl et al., 2000). O primeiro alvo do produto do gene Sry é o fator de transcrição Sox9. SRY ativa a síntese de SOX9 em precursores de células de Sertoli, e estes últimos, por sua vez, ativam outros genes envolvidos na diferenciação dessas células: Fgf9 (fator 9 de crescimento de fibroblasto) e prostaglandina D sintase. Esses eventos estimulam a diferenciação a e proliferação de células de Sertoli, sendo imprescindíveis para a formação do testículo (Ungewitte, Yao, 2013). Assim como as células do epitélio celômico, as células dos mesonefron também migram para o interior do testículo em formação. As primeiras dão origem às múltiplas linhagens celulares (tais como as células de Sertoli e células intersticiais); as segundas, exclusivamente às células endoteliais (Karl, Capel, 1998; Cool et al., 2008; Combes et al., 2009).

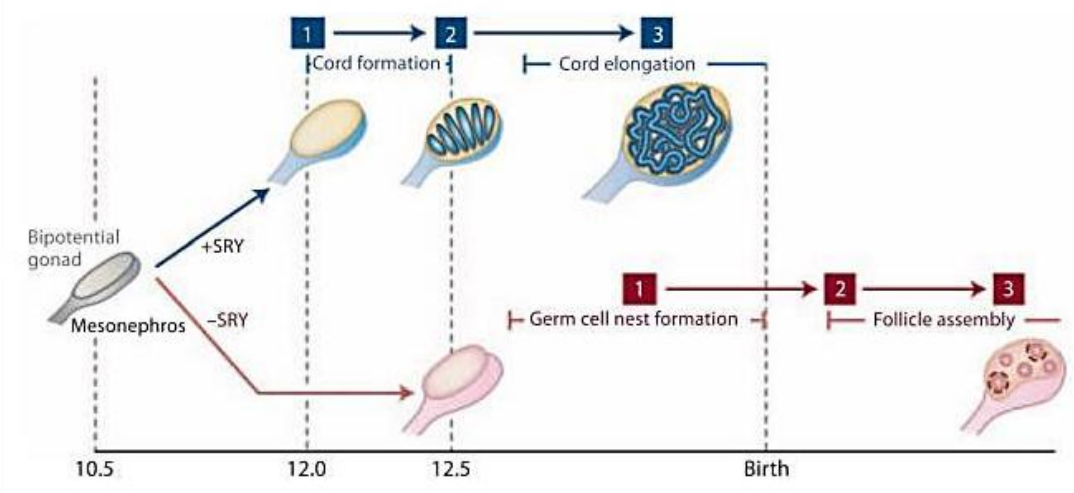

Figura 1. Cronograma dos principais eventos celulares na morfogênese do testículo e do ovário. Durante a embriogênese do camundongo, a gônada bipotencial é formada em torno de 10,5 dpc (dias pós-concepção), a partir da expressão do gene Sry (representação em azul), desencadeando o primeiro passo da morfogênese do testículo (1), a especificação da linhagem de células de Sertoli. Por volta de 12,5 dpc, o testículo compartimentalizou-se para formar estruturas de cordões cercadas por uma lâmina basal (2). Este passo requer o recrutamento de células mioides peritubulares, que envolvem os cordões. O estágio final (3) do desenvolvimento do testículo é o crescimento, em extensão, do cordão testicular, com a transformação de simples voltas em um sistema de cordões convolutos. Na gônada XX, o gene Sry está ausente (representação em rosa) e os eventos envolvidos na morfogênese testicular não ocorrem. A especificação da linhagem de células da granulosa define o 1º passo da organogênese do ovário (1). As células germinativas femininas, dentro do nicho de células germinativas, começam a entrar em meiose a partir de 13,5 dpc e param no estádio de diplóteno da meiose I, iniciado no dia 17,5 pc (pós-concepção). Os nichos de células germinativas desfazem-se logo após o nascimento, (2) quando as células da granulosa circundam ovócitos individuais para formar os folículos primordiais. A ativação folicular compartimentaliza o ambiente das células somáticas durante a primeira semana de vida pós-natal (3), acompanhada pelo recrutamento de células da teca para o folículo primário. Extraído de Ungewitte, Yao (2013).

Além das células de Sertoli, as células mioides peritubulares são essenciais para a formação de cordões seminíferos funcionais. A diferenciação dessas células ocorre devido ao fator DHH (*desert hedgehog* ou ouriço-do-deserto), secretado pelas células de Sertoli (Pierucci-Alves et al., 2001). As células peritubulares são células fusiformes que delimitam o cordão seminífero, formando, assim, um microambiente fechado, necessário para o bom desenvolvimento das células germinativas (Hadley et al., 1985; Skinner et al., 1985). As células de Sertoli, em resumo, estão envolvidas na primeira onda de expansão e crescimento dos cordões seminíferos. Entretanto, são as células de Leydig que controlam a expansão dos cordões durante o desenvolvimento fetal tardio. A inativação da activina A – um membro da família TGFβ – ocorrida especificamente nas células de Leydig fetais, não afeta a formação inicial dos cordões. Por outro lado, o crescimento destes é afetado após 15,5 dpc (dias pós-concepção), em camundongos (Archambeault, Yao, 2010).

Além do papel preponderante no desenvolvimento fetal dos cordões testiculares, as células de Leydig têm como principal função a síntese de esteroides (Pelliniemi, Niemi, 1969). Os andrógenos sintetizados pelas células de Leydig fetais são imprescindíveis para a diferenciação dos ductos mesonéfricos e atuam juntamente com o fator INSL3 (peptídeo 3 semelhante à insulina) – também sintetizado por elas – da descida testicular (O'Shaughnessy et al., 2006). As células de Leydig diferenciam-se a partir de células tronco mesenquimais, localizadas na região intersticial da gônada (Byskow, 1986). A citodiferenciação desse tipo celular inicia-se nos: aumento do volume citoplasmático, desenvolvimento do retículo endoplasmático liso, aumento do número e do tamanho das mitocôndrias, crescimento do núcleo e acúmulo de gotículas lipídicas, necessárias para a síntese de esteroides. A diferenciação morfológica e funcional das células de Leydig relaciona-se à habilidade de produzir testosterona no dia 15,5 dpc, em ratos (Warren et al., 1973; Habert, Picon, 1984), e entre a 6ª e 7ª semanas de gestação, em humanos (Tapanainen et al., 1981). Tanto em humanos como em outros mamíferos, o hormônio luteinizante (LH) não controla a diferenciação inicial das células de Leydig, uma vez que a produção de esteroides pelo testículo fetal é anterior à secreção de LH pela hipófise (Reyes et al., 1989). Entretanto, a partir de 18 dpc, em ratos, o desenvolvimento das células de Leydig mantém-se sob o controle do LH fetal (Eguchi et al., 1978; Habert, Picon, 1982). Em humanos, a gonadotrofina coriônica humana (hCG) produzida pela placenta não é necessária para a diferenciação inicial das células de Leydig. Após a fase preliminar de diferenciação, ela é requerida para produção suficiente de testosterona, necessária para a formação da genitália externa (Kremer et al.,

1995). Logo depois ao nascimento, ocorre o desenvolvimento de uma segunda onda de células de Leydig (Fouquet et al., 1984, Codesal et al., 1990; Prince, 1990). Em seguida, o número de células de Leydig diminui, de modo a que muito poucas permanecerão até o primeiro ano de vida, em humanos (Codesal et al., 1990; Prince 1990). Sendo assim, incluindo a onda púbere, existem três ondas de desenvolvimento de células de Leydig no homem (Lejeune et al., 1998). As células de Leydig adultas são derivadas de células precursoras indiferenciadas, e não de células de Leydig pré-existentes (Ge et al., 1996). Essas células percursoras se proliferam intensamente no período neonatal e, em torno do dia 14 pc, no rato, passam a ser denominadas células de Leydig progenitoras. Tais células fusiformes expressam marcadores específicos das células de Leydig, incluindo a 3β-hidroxiesteroide desidrogenase (3β-HSD), receptor de LH e de andrógeno (Hardy et al., 1990; Shan, Hardy, 1992). Aos 28 dpp (dias pós-parto) as células progenitoras assumem um formato redondo e a atividade das três enzimas esteroidogênicas P450scc (enzima de clivagem da cadeia lateral do colesterol), P450c17 (17alfa-hidroxilase/17, 20-liase) e 3β-HSD aumenta entre os dias 28 e 56 (Shan, Hardy, 1992; Dupont et al., 1993; Shan et al., 1993). A enzima 17β-HSD (17β-hidroxiesteroide desidrogenase), que catalisa a síntese de testosterona a partir da androstenediona, não é marcante até os 35 dias de vida. No rato, as células de Leydig imaturas se dividem apenas uma vez, entre os dias 28 e 56, antes de se diferenciarem em células de Leydig adultas (Lejeune et al., 1998).

Por muitos anos acreditou-se que o desenvolvimento da gônada feminina era passivo. Na ausência do cromossomo Y, da expressão do gene Sry e, consequentemente, da secreção de AMH e de testosterona, os ductos de Wolff sofreriam regressão passiva; enquanto o desenvolvimento, também passivo, dos ductos de Müller levaria ao desenvolvimento das tubas uterinas, do útero e do terço superior da vagina (Carlson, 1994). Não obstante, hoje os genes envolvidos na diferenciação da gônada feminina foram identificados – Wnt4 (membro 4 da família Wnt), β-catenina e Rspo-1 (R-espondina 1). Estes também inibem a expressão de Sox9 nos ovários em formação (Baillet et al., 2011; Biason-Lauber, 2010).

I. 2. Os gonócitos

A espermatogênese é um processo complexo de diferenciação celular durante o qual as células germinativas se desenvolvem em gametas masculinos ou espermatozoides. O desenvolvimento ocorre no interior dos túbulos seminíferos e compreende três etapas: (1) diferenciação das espermatogônias e sua proliferação mitótica; (2) meiose dos espermatócitos e (3) conversão de espermátides haplóides em espermatozoides (Leblond, Clermont, 1952). O

início da espermatogênese e sua manutenção em níveis ótimos, após a maturação testicular, são fundamentais para a capacidade reprodutiva da espécie. Por sua vez, a espermatogênese depende do desenvolvimento adequado do testículo, no período embrionário, e da diferenciação pós-natal de suas populações celulares.

As CGPs (células germinativas primordiais) originam-se no epiblasto extra-embrionário e migram através do intestino posterior e do mesentério dorsal (De Sousa Lopes et al., 2007). A invasão do mesoderma intermediário, situado ventro-lateralmente ao mesonefron por essa população de células migratórias, leva à formação do primórdio gonadal, também conhecido como crista genital (Fig. 2), com capacidade de originar o testículo ou o ovário (Magre, Jost, 1980; McLaren, 1999).

Após alcançarem as cristas genitais, as CGPs são aprisionadas no interior dos cordões sexuais primitivos, passando a ser designadas gonócitos (Clermont, Perey, 1957; Magre, Jost, 1980). Desse modo, o termo gonócito foi originalmente proposto por Clermont e Perey (1957) para designar as células germinativas originadas no período fetal, a partir do momento em que estas ocupam o interior dos cordões sexuais primitivos até sua diferenciação neonatal em espermatogônias. Entretanto, Cooke et al. (1993) propuseram que a expressão de fosfatase alcalina é um indicador da transição das CGPs para gonócitos. Entre os dias 7,5 – 8 dpc, em roedores, e na terceira semana de gestação, em humanos, as CGPs apresentam forte marcação à fosfatase alcalina. Essas células perdem a expressão de fosfatase alcalina em torno do dia 14,5 pc, em roedores (Richards et al., 1999), passando a ser denominadas gonócitos (Culty, 2009).

Apesar de o desenvolvimento das células germinativas nos períodos fetal e neonatal parecerem bem mais simples e homogêneo em comparação às outras populações de células germinativas encontradas durante a puberdade e a fase adulta, tal simplicidade é apenas aparente (Culty, 2009). De acordo com Nagano et al. (2000), ao invés de estarem sincronizados em cada período do desenvolvimento, os gonócitos apresentam sobreposição de subpopulações com a presença, em um mesmo corte de cordão seminífero, de gonócitos quiescentes e mitóticos, bem como gonócitos localizados na base ou no centro dos cordões – os primeiros estarão em processo de diferenciação; enquanto os últimos, na fase pré-migratória ou destinados à apoptose. Há muita controvérsia na literatura em relação à nomenclatura desse tipo celular – além de gonócito, os termos pré ou pró-espermatogônia também foram utilizados (Culty, 2009). Outros autores consideram o termo pró-

espermatogônia mais apropriado para descrever a fase de transição entre os gonócitos e as espermatogônias (Ohbo et al., 2003; Pinto et al., 2010).

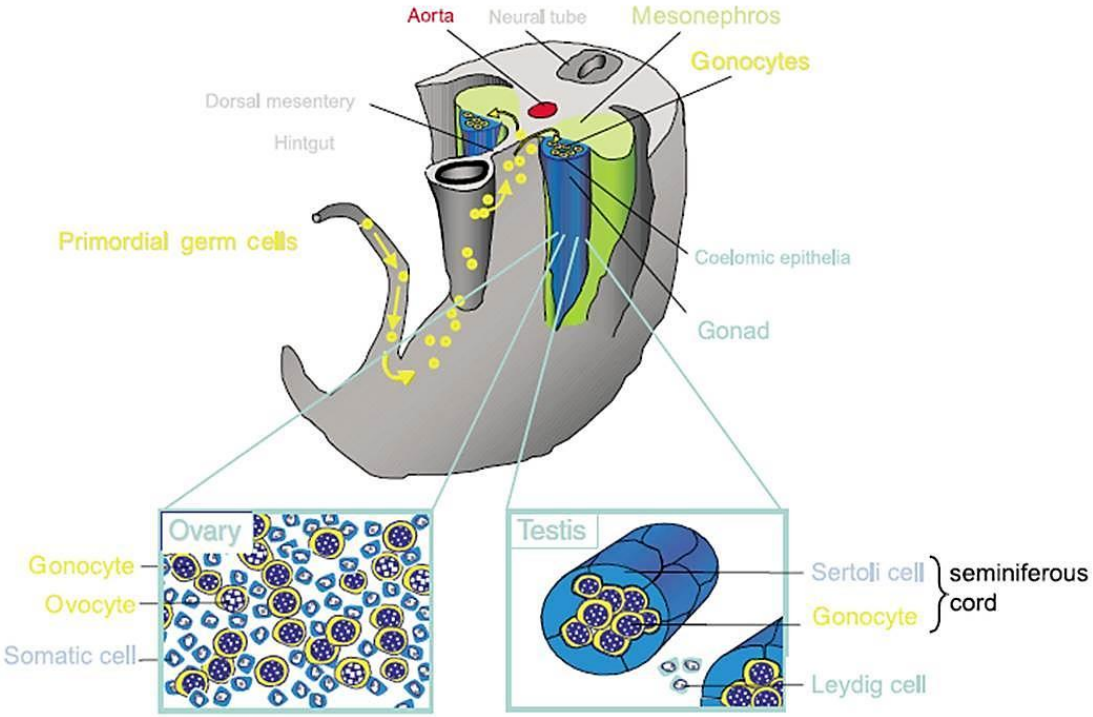

Figura 2. Parte posterior de um embrião mostrando o desenvolvimento da gônada. A gônada aparece pela primeira vez como um inchaço, idêntica em ambos os sexos e originada a partir da proliferação de células dos mesonefron e do epitélio celômico, sendo colonizada pelas células germinativas primordiais. Depois disso, a gônada segue um destino ovariano ou testicular. No ovário, tanto as células somáticas como as germinativas permanecem desorganizadas. No testículo, ao contrário, as células de Sertoli rodeiam os gonócitos para formar os cordões seminíferos. Extraído de Rouiller-Fabre et al. (2003).

Os gonócitos, assim como as CGPs, são células muito volumosas, cujo núcleo proeminente contém um ou dois nucléolos cercado por um citoplasma em forma de anel (Clermont, Perey, 1957; Baillie, 1964; Culty, 2009). Apesar das semelhanças morfológicas, estudos *in vitro* têm demonstrado diferenças funcionais entre esses dois tipos celulares. Os gonócitos, por exemplo, não podem sobreviver sem a presença das células de Sertoli; as CGPs, por sua vez, podem ser co-cultivadas com outros tipos de células somáticas (Resnick et al., 1992; van Dissel-Emiliani et al., 1993). A diferenciação das CGPs em gonócitos marca a transição de células de potencial múltiplo para células unipotentes, relacionadas ao desenvolvimento da linhagem germinativa masculina (Matsui, et al., 1992; de Rooij, 1988). Os gonócitos neonatais são os precursores das células tronco espermatogoniais (SSCs) e das

espermatogônias do tipo A da primeira onda espermatogênica (Yoshida et al., 2006; Manku, Culty, 2015). Embora tenham sido caracterizados há muito tempo, a biologia dessas células e os mecanismos que regulam sua diferenciação ainda são pouco compreendidos, em comparação a outros tipos celulares do testículo (Culty, 2009; Manku et al., 2016).

Os eventos envolvidos na diferenciação dos gonócitos têm sido estudados em roedores de laboratório, como o camundongo e o rato. Estudos dessa natureza indicam que os roedores mencionados apresentam duas fases de proliferação: uma, no período fetal, e outra, no neonatal, intercaladas por um período de quiescência (Culty, 2009). Em ratos, a primeira fase proliferativa ocorre entre 13,5 e 17,5 dpc, ao passo que o período de quiescência se estende de 17,5 dpc até o nascimento (Magre, Jost, 1980; Orth, 1982; McGuiness, Orth, 1992a, b). A atividade mitótica dos gonócitos é retomada entre os dias 1-4 pós-parto, quando também ocorre a sua diferenciação em espermatogônias A (Clermont, Perey, 1957; Orth, 1982; McGuiness, Orth, 1992a, b; Prépin et al., 1994; De Miguel et al., 1997; Boulogne et al., 1999). Em camundongos, os gonócitos proliferam-se no momento da formação dos cordões seminíferos, quando estes são cercados pelas células de Sertoli, entre os dias 11,5 e 12,5 pc até o dia 15,5 pc. O período de quiescência estende-se de 15,5 dpc até o nascimento, ocasião em que ocorre a segunda fase proliferativa deste tipo celular (Vergouwen et al., 1991; Vergouwen et al. 1993; Nagano et al., 2000). Os gonócitos desaparecem totalmente a partir do dia 10 pós-concepção, no rato (Clermont e Perey, 1957). Em outros roedores, como o hamster e o gerbilo, o padrão de diferenciação é similar, embora neste último eles sejam observados até o final da segunda semana de vida (Miething, 1992; Pinto et al., 2010). Em relação ao gerbilo da Mongólia, os eventos envolvidos na diferenciação pós-natal dessas células foram examinados por nosso grupo de pesquisa (Pinto et al., 2010) e está ilustrado na Figura 3. Essas informações básicas permitem que esse roedor seja utilizado para avaliações dos efeitos toxicológicos e ambientais sobre o desenvolvimento testicular.

Evento indispensável para a diferenciação dos gonócitos em espermatogônias, durante o período neonatal, é o seu reposicionamento (*relocation*); ou seja, a migração do centro para a base dos cordões seminíferos em desenvolvimento e a adesão à membrana basal (Roosen-Runge, Leik, 1968; Clark, Eddy, 1975; McGuiness, Orth, 1992a). Em ratos, o reposicionamento ocorre um dia após o início da proliferação neonatal, e em camundongos, em torno de 2,5 dpp, o que sugere que esses eventos são independentes e, provavelmente, regulados por mecanismos distintos (McGuinness, Orth, 1992a, Culty, 2009). Na maioria dos

roedores o período proliferativo neonatal coincide com um pico de morte dos gonócitos por apoptose (Miething, 1992; Boulogne et al., 1999).

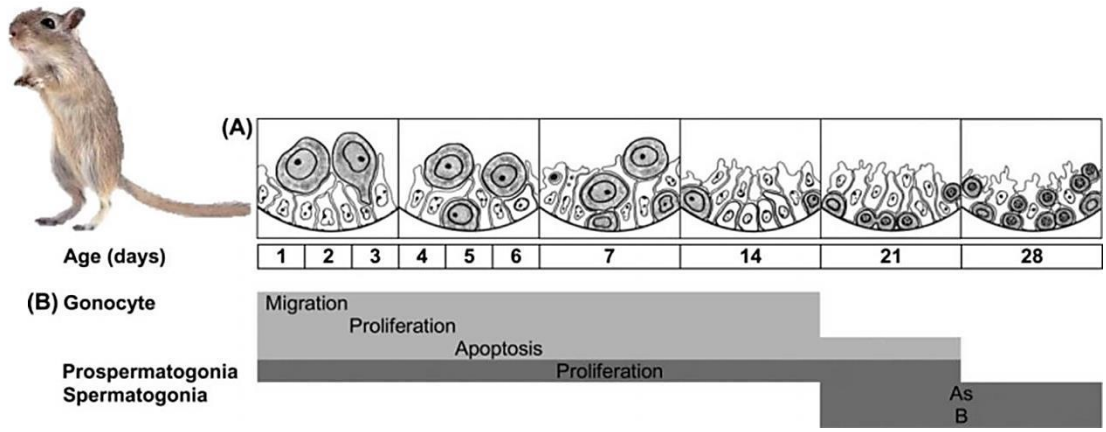

Figura 3. Resumo dos principais eventos no desenvolvimento pós-natal do gerbilo da Mongólia. (A) Ilustração esquemática das alterações histológicas no epitélio seminífero. As estruturas em cinza representam as células da linhagem germinativa e as estruturas em branco, as células de Sertoli. (B) A duração dos eventos referentes aos gonócitos e às espermatogônias está indicada pelas barras. Adaptado de Pinto et al. (2011).

Existem sólidas evidências sobre o papel dos andrógenos no desenvolvimento das células germinativas durante o período fetal. Alguns casos de insensibilidade a andrógeno e de baixos níveis desse hormônio estão associados a um alto risco de câncer testicular, devido ao desenvolvimento alterado dos gonócitos (Skakkeback et al., 2001; Fisher, 2004). Também foi constatada que a exposição de ratas prenhes a anti-andrógeno, durante o período de 8 a 15 dpc, afetou a espermatogênese dos adultos (Anway et al., 2005). No entanto, o mesmo tratamento entre 15 e 20 dpc não apresentou nenhum efeito (Cupp et al., 2003; Uzumco et al., 2004). Merlet et al. (2007) observaram um aumento no número de gonócitos devido a maior atividade proliferativa em camundongos mutantes com síndrome da feminilização testicular (Tfm), os quais não apresentam receptor de andrógenos (AR). Em adição, o cultivo de gonócitos fetais na presença de dihidrotestosterona (DHT) resultou na diminuição de sua atividade proliferativa. Esses autores também demonstraram a presença de mRNA para AR, bem como de AR funcional nessas células. Portanto, segundo Merlet et al. (2007), os gonócitos têm um período crítico de sensibilidade a andrógenos durante sua fase de proliferação. Vários estudos evidenciam que a ação da testosterona sobre a espermatogênese é indireta e mediada por fatores parácrinos, produzidos pelas células de Sertoli, visto que as várias fases de diferenciação das células germinativas, ao longo da espermatogênese, são AR-

negativas (Bremner et al., 1994; Vornberger et al., 1994; Matsumiya et al, 1999). No caso do gerbilo da Mongólia, a transição de gonócitos para espermatogônias representa uma fase de perda de sensibilidade androgênica no decorrer do período neonatal (Pinto et al., 2010).

I. 3. Os ftalatos

Os desreguladores endócrinos (DEs) são agentes exógenos que interferem no funcionamento do sistema endócrino por provocarem diminuição, aumento ou substituição de hormônios naturais, ou ainda por bloquearem a ação destes (Santamarta, 2001). Os ésteres de ftalato são DEs usados para manter a cor e o aroma de uma grande quantidade de produtos de consumo, tais como solventes de tinta, cola, repelentes, lubrificantes e adesivos, podendo também ser encontrados em produtos de cuidado pessoal. No último caso mencionado, o dietil ftalato (DEP), o di-n-butil ftalato (DBP) e o benzil butil ftalato (BBzP) são abundantemente encontrados em loções para o corpo, gel, xampus e desodorantes (Hauser et al., 2004). Para prever uma possível exposição humana aos ftalatos presentes em cosméticos, Koo e Lee (2004) mediram os níveis de di-(2-etilhexil) ftalato (DEHP), DEP, DBP e BBzP em 102 marcas de spray para cabelo, perfumes, desodorantes e esmaltes. O DBP foi detectado em 90% dos esmaltes e 26% dos perfumes, e o DEP em 57% dos perfumes e em 25% dos desodorantes analisados. Apesar de frequentes, os ftalatos foram detectados em níveis relativamente pequenos nos cosméticos. Entretanto, considerando-se a exposição total a várias fontes desses compostos essa taxa deve ser bem maior (Koo, Lee, 2004). Por garantirem flexibilidade e maleabilidade aos plásticos, estes aditivos químicos também são comumente utilizados na fabricação de brinquedos, embalagens de alimentos e equipamentos médicos (Kavlock et al., 2002; Loureiro, 2002). Apesar da volatilidade dos ftalatos ser relativamente baixa, Rudel et al. (2003) mostraram que estes estão presentes no ar do interior das residências. Sendo assim, o contato com esses compostos químicos pode ocorrer por via oral, dermatológica ou respiratória (Koch et al., 2006). Em um estudo epidemiológico, Hauser et al. (2004) demostraram que os medicamentos também podem ser uma fonte de exposição aos ftalatos. Segundo os autores, o uso de Asacol (medicamento usado para o tratamento de colite ulcerativa) levou a uma concentração de DBP mais de duas ordens de magnitude maior que o percentual considerado normal para homens, na urina de um dos voluntários participante do estudo (Hauser et al., 2004). O revestimento entérico – técnica utilizada para evitar que a cápsula dissolva-se no estômago, fazendo com que o medicamento seja liberado no intestino –

é uma fonte de exposição a diversos polímeros que contêm plastificantes, dentre eles o DEP e o DBP (Harris, Ghebre-Sellassie, 1989; Frohoff-Hulsmann et al., 1999).

O DEHP é o éster de ftalato mais comumente usado em produtos farmacêuticos e equipamentos médicos (Testa et al., 2012). Após a exposição, o DEHP é rapidamente hidrolisado à mono-(2-etilhexil) ftalato (MEHP), provavelmente a molécula ativa (Gray, 1986). Posteriormente, o MEHP é oxidado nas posições ω e ω-1, levando à formação de uma série de metabólitos secundários (Albro, 1986; Koch et al., 2003, 2006) (Fig. 4A). Tais metabótitos podem, por sua vez, em seguida à reação de glucuronidação, ser excretados na urina (Fig. 4B). Koch et al. (2004) avaliaram o metabolismo humano do DEHP ulteriormente à administração de uma única dose oral de 48,1 mg em um voluntário do sexo masculino. Os autores monitoraram a excreção de três metabólitos: mono-(2-etil-5-hidroxihexil) ftalato (5OH-MEHP), mono-(2-etil-5-oxohexil) ftalato (5oxo-MEHP) e MEHP, na urina e no soro do voluntário. Após 44 horas, aproximadamente 50% do DEHP ingerido foi eliminado na urina, sendo que, desse total, 24,4% e 14,9% sob a forma de 5OH-MEHP e de 5oxo-MEHP, respectivamente, e apenas 7,3% sob a forma de MEHP. Todavia, o MEHP foi o principal metabólito encontrado no soro. Tal fato se deve ao menor caráter hidrofílico do MEHP, que tende a se acumular no sangue, sendo de longe o ftalato mais presente no plasma sanguíneo (Koch et al., 2004). Em geral, há pouca retenção do DEHP ingerido no organismo, pois a maior parte é eliminada na urina (Tanaka et al., 1975, Koch et al., 2004). Entretanto, esse ftalato pode ser encontrado em diversos órgãos e tecidos como fígado, rim, tecido adiposo e testículo (Tanaka et al., 1975; Loureiro, 2002). Neste último, os ftalatos podem afetar tanto as células germinativas como as células somáticas e a produção de testosterona (Chauvigné et al., 2009).

Uma vez que os ésteres de ftalato podem atravessar a placenta e também estarem presentes no leite materno, grande atenção tem sido voltada aos efeitos sobre a prole, após a exposição das mães a esses compostos (Shea, 2003; Huang et al., 2009; Scarano et al., 2009; Wittassek et al., 2009; Li et al., 2013). Segundo Chauvigné et al. (2009), testículos fetais de ratos expostos ao MEHP apresentaram diminuição do número de gonócitos, diminuição do índice de mitose e aumento da apoptose das células germinativas, alteração da morfologia dos túbulos seminíferos e inibição da produção de testosterona. Não obstante, nenhum efeito deletério foi observado depois da exposição ao DEHP, indicando que as gônadas de embriões de 14,5 dpc, utilizadas nos experimentos, não expressam a esterase nem a enzima citocromo P450, ambas envolvidas no metabolismo do ftalato. Esses resultados também reforçam a

hipótese de que o MEHP é o metabólito ativo do DEHP responsável por desordens testiculares tanto em estudos *in vitro* (Chauvigné et al., 2009) como em estudos *in vivo* (Foster et al., 2001). Tais efeitos adversos também confirmam que a gestação e a lactação são períodos críticos de sensibilidade à exposição ao DEHP devido à ação anti-androgênica desse composto (Parks et al., 2000; Foster et al., 2001; Andrade et al., 2006; Christiansen et al., 2010).

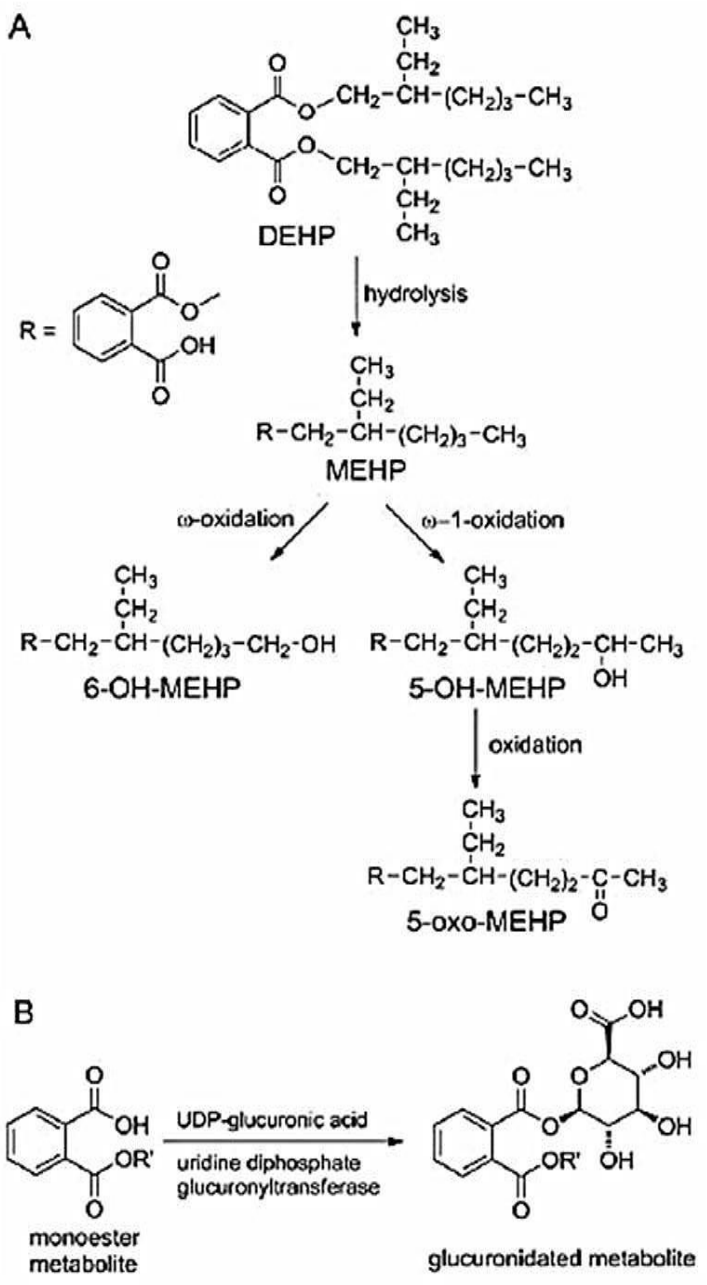

Figura 4. Metabolismo do DEHP. (A) via hidrolítica/oxidativa levando à formação do MEHP e metabólitos secundários. (B) Formação de conjugados glucurônicos de metabólitos do DEHP. Extraído de Testa et al. (2012).

O sistema de cultura organotípica tem se tornado um importante método alternativo para o estudo dos efeitos *in utero* do MEHP tanto em roedores como em humanos (Lambrot et al., 2009; Lehraiki et al., 2009, Muczynski et al., 2012a). Nesse sistema, inicialmente proposto para o estudo em rato (Habert et al., 1991), os testículos são cultivados sobre um filtro flutuante em meio sintético sem soro, sem hormônios e sem fatores biológicos (Livera et al., 2000, 2001, 2002). A arquitetura e a comunicação intercelulares são preservadas e os principais tipos celulares do testículo desenvolvem-se normalmente, pelo período de uma a duas semanas, no caso do rato. Outro ponto a ser destacado é a sobrevivência das células germinativas por um tempo superior a 24 horas (Livera et al., 2006). Sendo assim, esse sistema permite investigar o efeito direto de um determinado xenobiótico – dentre eles os ésteres de ftalato – sobre o desenvolvimento testicular, em condições controladas, o que não pode ser facilmente monitorado *in vivo*.

Considerando que o receptor de estrógeno do tipo β (ERβ) e o AR estão envolvidos no desenvolvimento dos gonócitos (Delbès et al., 2004; Merlet et al., 2007), os efeitos dos ftalato sob o testículo fetal/neonatal foram inicialmente descritos por sua ação anti-androgênica e anti-estrogênica. Entretanto, Lehraiki et al. (2009) verificaram que camundongos de 13,5 a 18,5 dpc, deficientes em receptores de estrógeno (ERαKO, ERβKO) ou andrógeno (Tfm), também exibiram redução no número de gonócitos após exposição ao MEHP, levando à conclusão de que este ftalato interfere no número de células germinativas, sem envolver direta ou indiretamente os receptores de estrógeno ou o receptor de andrógeno. Além dos prejuízos à diferenciação do sistema reprodutor de roedores, o MEHP também é prejudicial ao desenvolvimento do testículo humano. Lambrot et al. (2009) conduziram um estudo pioneiro demostrando o efeito deletério do ftalato sobre as células germinativas de testículo de fetos durante o primeiro trimestre de gestação (7 a 12 semanas). Os autores observaram uma diminuição no número de gonócitos devido ao aumento da apoptose, sem alteração na proliferação dessas células. No entanto, ao contrário do verificado para roedores, o MEHP não afetou a secreção de testosterona pelo testículo. Um aspecto importante é a interferência espécie-específica do ftalato sobre as células de Leydig fetais e, consequentemente, sobre a esteroidogênese. Portanto, comparando-se os efeitos de diferentes desreguladores endócrinos sobre os testículos fetais de humanos, ratos e camundongos, Habert et al. (2014) concluíram que o efeito anti-androgênico dos ftalatos, encontrado para o testículo de ratos, não existe para a espécie humana. Enquanto os ftalatos prejudicam o desenvolvimento das células germinativas de humanos (Lambrot et al., 2009) e de camundongos (Lehraiki et al., 2009),

sem alterar a produção de testosterona, a exposição intrauterina de ratos a esses compostos causa uma redução nos níveis de andrógenos (Hallmark et al., 2007).

Além dos efeitos deletérios provocados pelos metabólitos ativos do DEHP, grande atenção tem sido voltada para o potencial do DBP (Fig. 5) sobre o desenvolvimento cerebral (Li et al., 2013) e reprodutivo (Foster et al., 2001), malformações e toxicidade reprodutiva e morte fetal (Shea, 2003). Estudos têm revelado que esse ftalato induz à toxicidade testicular – esta parece estar relacionada à idade dos indivíduos –, sendo os adultos menos susceptíveis a seus efeitos em comparação aos jovens púberes (Foster et al., 1980; Sjoberg et al., 1986).

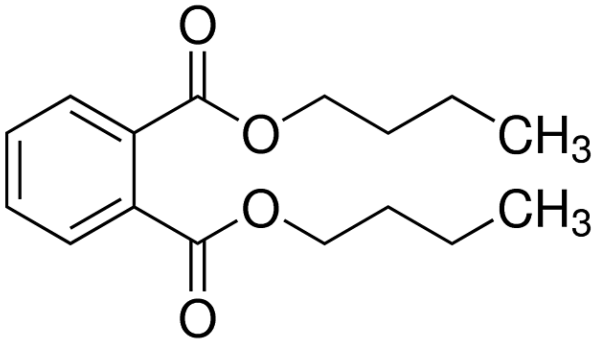

Figura 5. Estrutura química do di-n-butil ftalato (DBP).

Efeitos adversos da exposição ao DBP, no decurso do período embrionário, são o aumento da proliferação das células de Leydig, presença de gonócitos multinucleados (GMNs) no interior dos cordões seminíferos e diminuição dos níveis de testosterona (Mylchreest et al., 1999). A observação de gonócitos multinucleados foi interpretada pelos autores como uma iniciação inapropriada da divisão celular e o aumento do número de células de Leydig como um mecanismo compensatório, devido à diminuição dos níveis de testosterona no testículo fetal. Entretanto, com o intuito de determinar o período de formação dos GMNs e compreender se este está relacionado à divisão celular, Spade et al. (2015) expuseram ratas a uma dose única de DBP (500 mg/Kg) nos dias 17 e 18 de gestação. As ratas foram eutanasiadas após 24 horas, ou expostas a uma segunda dose e eutanasiadas após 48 horas. O DBP induziu uma formação significante de GMNs após 24 horas, somente para a exposição no dia 18 pós-parto. Os GMNs marcados pelo TUNEL foram raros; em relação à proliferação, dos 606 GMNs, nos cortes imunomarcados com BrdU (5-bromo-2'-desoxiuridina), apenas um apresentou forte marcação nuclear, confirmando o mecanismo não proliferativo de formação dos GMNs (Spade et al., 2015).

Os efeitos deletérios do DBP também são conhecidos para o período pré-pubere e para a vida adulta. Moody et al. (2013) expuseram camundongos a doses crescentes de DBP (1-500 mg/kg/dia) do dia 4 aos dias 7, 14 ou 21 dpp. Para o estudo dos efeitos do ftalato na vida adulta, os animais de 21 dias foram mantidos sem o xenobiótico até a oitava semana. Uma redução crescente do peso testicular foi observada nos animais de 14 dias, para as doses de 50, 100, 250 e 500 mg de DBP/kg/dia. Surpreendentemente, ao contrário do observado para o testículo fetal humano e de roedores (Muczynski et al., 2012b), a apoptose não foi uma consequência significante da exposição ao DBP no período pré-pubere. Entretanto, a maturação das células de Sertoli foi afetada. A reações imunohistoquímica para o fator de transcrição SOX9, um marcador de núcleo das células de Sertoli (Fröjdman et al, 2000), indicou uma localização basal dos núcleos desse tipo celular no grupo controle e uma localização central, nos grupos administrados com menor concentração do ftalato (1-10 mg de DBP/Kg/dia). Os animais submetidos a uma concentração maior (100-500 mg de DBP/kg/dia) apresentaram uma desorganização substancial das células de Sertoli, além de clusters desse tipo celular nos cordões/túbulos seminíferos. E a dose mais alta de DBP (500 mg/kg/dia) provocou o atraso da primeira onda de espermatogênese e a redução dos níveis séricos de testosterona nos animais de 14 dpp. Em estudo recente, Bielanowicz et al. (2016) demostraram que os efeitos da exposição pré-pubere ao DBP sobre o desenvolvimento e a função dos testículos se estendem para funções não reprodutivas. Camundongos adultos expostos a uma baixa dose de ftalato (10 mg/Kg/dia), do dia 4 ao dia 14 pc, apresentaram redução no conteúdo mineral ósseo (CMO) e na densidade mineral óssea (DMO). Contudo, a administração de doses maiores (100-500 mg/kg/dia) não alterou o CMO nem o DMO, mas induziram o aumento do número de células de Leydig, que influenciam o metabolismo dos ossos (Ferlin et al., 2013), e a densidade de células intersticiais, preservando a produção de testosterona. Tais resultados apontam para uma alteração compensatória das células testiculares envolvidas na manutenção do osso (Bielanowicz et al., 2016).

Além dos efeitos deletérios observados nos estudos *in vivo*, Chen et al. (2013) demostraram, em estudo *in vitro*, que a secreção de testosterona é estimulada em baixas doses (0,01 µmol/L) e inibida em altas doses (100 µmol/L) de DBP. Esse fato relaciona-se à diminuição dos níveis de RNAm das enzimas esteroidogênicas P450scc, P450c17, 3β-HSD e da proteína INSL3, um importante marcador da diferenciação das células de Leydig.

I. 4. O ácido docosahexaenoico (DHA)

O ácido docosahexaenoico (DHA, 22:6 n-3) é um ácido graxo polinsaturado de cadeia longa (PUFA) pertencente à família ômega-3 (Fig. 6) e derivado do ácido alfa-linolênico (AAL, 18:3 n-3). A síntese de DHA ocorre no fígado, após sucessivas dessaturações e alongamento do AAL (Fig. 7), uma cascata metabólica considerada muito fraca em humanos (Martin et al., 2006; Picq et al., 2010).

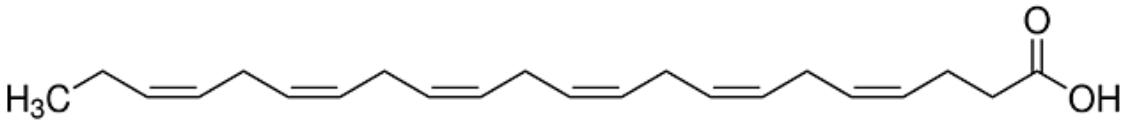

Figura 6. Estrutura química do ácido docosahexaenoico (DHA).

O transporte de ácidos graxos, incluindo o DHA, é feito pela albumina sérica, que também se liga à lisofosfolipídeos, principalmente a lisofosfatidilcolina (LysoPC) (Picq et al., 2010). No fígado, o DHA complexado à fosfatidilcolina (PC-DHA) pode ser hidrolizado a LysoPC-DHA, numa reação catalisada pela enzima fosfolipase A1, ou ser liberado e hidrolizado à LysoPC-DHA pela lipase endotelial. Tanto o DHA esterificado a LysoPC como a forma não esterificada podem ser transportados pela albumina (Picq et al., 2010). Em estudo realizado por Thies et al. (1994) foi demonstrado que a incorporação de DHA pelo cérebro de ratos jovens (20 dias de idade) sob a forma de LysoPC-DHA foi 10 vezes maior que sob a forma não esterificada (4-5% de LysoPC-DHA contra 0,3-0,4% de DHA), ambas ligadas à albumina. No entanto, para o coração, o fígado e os rins não foi observada preferência entre as duas formas de DHA, sendo que fígado e coração acumularam mais DHA não esterificado que a forma LysoPC-DHA (Thies et al., 1994). Em humanos, o acúmulo de DHA pelas plaquetas sanguíneas ocorre principalmente sob a forma não esterificada ligada à albumina sérica, enquanto o DHA ligado à fosfatidilcolina é a principal fonte de DHA das células vermelhas do sangue (Brossard et al., 1997).

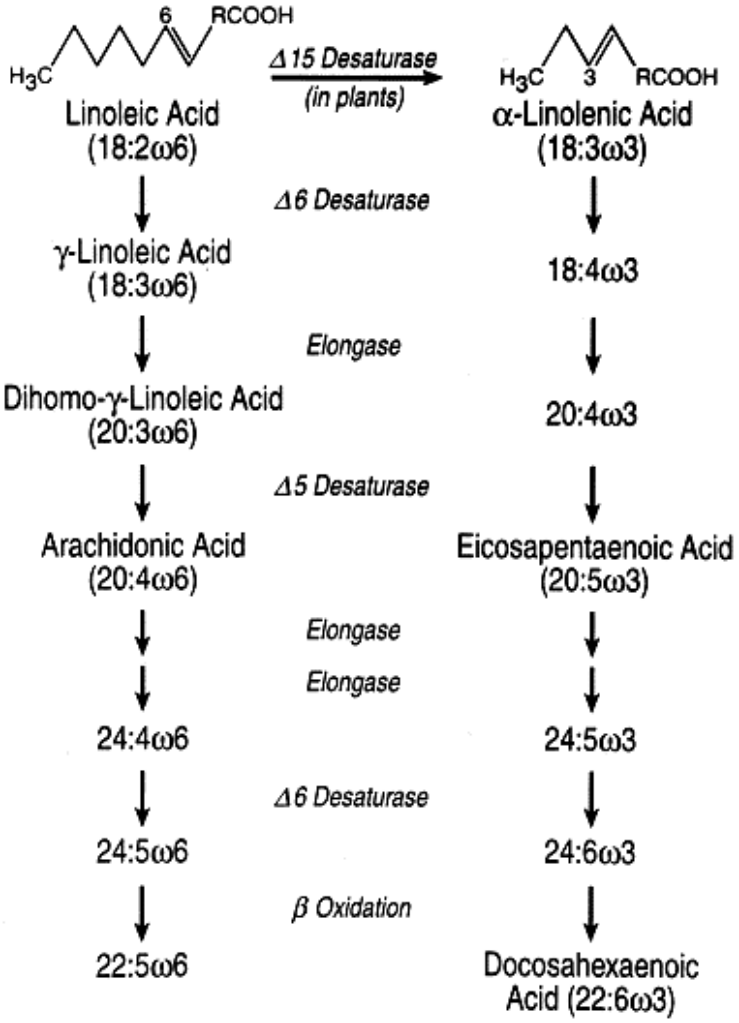

Figura 7. Via para a síntese de ácidos graxos polinsaturados. Extraído de Teitelbaum, Allan Walker (2001).

Por ser altamente insaturado, o DHA é facilmente oxigenado por diversas lipoxigenases para produzir lipoxinas (Aveldano, Sprecher, 1983). Dentre os derivados da oxidação do DHA, os mais bioativos são as resolvinas, que atuam na resolução de processos inflamatórios e a protectina D1 e a neuroprotectina D1, que apresentam potencial anti-inflamatório e neuroprotetor, respectivamente (Hong et al., 2003; Bannenberg et al., 2005; Bazan et al., 2005). Quando ocorre trauma ou infecção microbiana é desencadeada uma resposta do sistema imune – denominada resposta inflamatória – para eliminar os micro-organismos invasores e controlar efeitos danosos ao tecido. Resolução é o nome dado ao processo de recuperação do tecido e retorno à homeostase, ao término do processo inflamatório (Barbalho et al., 2011). Durante o processo de resolução, os níveis de citocinas pro-inflamatórias diminuem e ocorre uma mudança na síntese de mediadores lipídicos derivados de ácidos graxos polinsaturados ômega-3 (ácido eicosapentanoico - EPA e ácido

docosahexaenoico – DHA), que por sua vez mudam a conversão enzimática de fatores pro-inflamatórios (leucotrienos e prostaglandinas) para fatores pró-resolução (lipoxinas, resolvinas e protectinas) (Fig. 8). Muitas classes de resolvinas já foram caracterizadas, mas atualmente estas foram divididas em duas grandes classes: série-E, que corresponde à classe de resolvinas derivadas do EPA, e série-D, que englobe aquelas derivadas do DHA (Levy, 2010). Durante a síntese de resolvinas da série-D, o DHA serve de substrato para a enzima 15-lipoxigenase (ALOX15), que o tranforma em 17S-hidroxiperóxido-DHA (17S-H(p)DHA). Esse intermediário pode ser convertido em vários compostos bioativos, incluindo as resolvinas D1 (RvD1), D2 (RvD2), D3 (RvD3) e D4 (RvD4), e também a protectina D1 (PD1) e a neuroprotectina D1 (NPD1), nome dado à PD1 quando gerada em tecidos neurais (Hong et al., 2003; Serhan, Chiang, 2006). Os efeitos antioxidante e antiinflamatório dos ômegas 3 (EPA e DHA) já foram confirmados na prevenção de doenças inflamatórias como, por exemplo, a doença vascular aterosclerótica e a doença de Alzheimer (Chen et al., 2003; Hjorth et al., 2013).

Como já mencionado, a cascata metabólica que leva à conversão de AAL em DHA é muito fraca em humanos. Sendo assim, a suplementação desse ácido graxo deve ser feita através do consumo de alimentos ricos em ômega-3, como peixes de águas frias (salmão, atum e arenque, por exemplo), óleos de coco, soja e oliva, noz, linhaça e chia (Barbalho et al., 2011; Micha et al., 2014). Por ser de grande importância para a nutrição humana, a ingestão adequada de ω-3/ω-6 tem sido recomendada por órgãos de saúde de vários países (Martin et al., 2006). De acordo com a Organização Mundial de Saúde (WHO) / *Food and Agriculture Organization* (FAO) a ingestão de ω-3 e ω-6 deve ocorrer na razão de 5:1 a 10:1 (*World Health Organization. Joint Consultation: fats and oils in human nutrition. Nutrition Reviews*; v. 53, p. 202-205, 1995). Entretanto, a *Dietary Reference Intakes* (DRI) estabelece níveis de ingestão para os ácidos graxos ω-3 e ω-6 individualmente, e a recomendação de ingestão diária de ambos varia conforme o sexo, a idade e os estados fisiológicos, como gestação e lactação. Para homens entre 19 e 50 anos de idade recomenda-se o consumo diário de 17 g de ω-6 e 1,6 g de ω-3, e para mulheres na mesma faixa etária a recomendação é de 12 g e 1,1 g de ω-6 e ω-3, respectivamente, por dia (*Institute of Medicine. National Research Council. Dietary Intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macrontrients). Washington (DC): National Academy Press; 2002*). Entretanto, Micha et al. (2014) demonstraram que a média global de consumo do ω-3, proveniente de frutos do mar, foi de 163 mg/dia, com uma grande variação nacional (de 5 a 3886 mg/dia). O

maior consumo ocorre nas ilhas Maldivas, Barbados, Seicheles e Islândia, bem como na Malásia, Tailândia, Dinamarca, Coreia do Sul e Japão. Em 45 dos 187 países avaliados o consumo médio foi ≥ 250 mg/dia. Todavia, 100 nações tiveram consumo diário muito baixo (≤ 100 mg), em geral nas regiões da África subsaariana e da Ásia, bem como na África do Norte/Oriente Médio. O consumo médio de ω-3 proveniente de plantas foi de 1371 mg/dia, e a variação entre os países também foi grande (de <100 a >3000 mg/dia). Os países com maior consumo desse ácido graxo foram Jamaica, China, Reino Unido, Tunísia, Angola, Senegal, Argélia, Canadá e Estados Unidos devido, provavelmente, a grande produção de linhaça (Canadá, China e Estados Unidos) e de canola (Canadá). Várias nações da América do Sul, tais como Brasil, Argentina, Paraguai e Uruguai também são grandes consumidores de ω-3 proveniente de plantas, potencialmente devido ao consume de castanhas e sementes ricas em AAL (Micha et al., 2014).

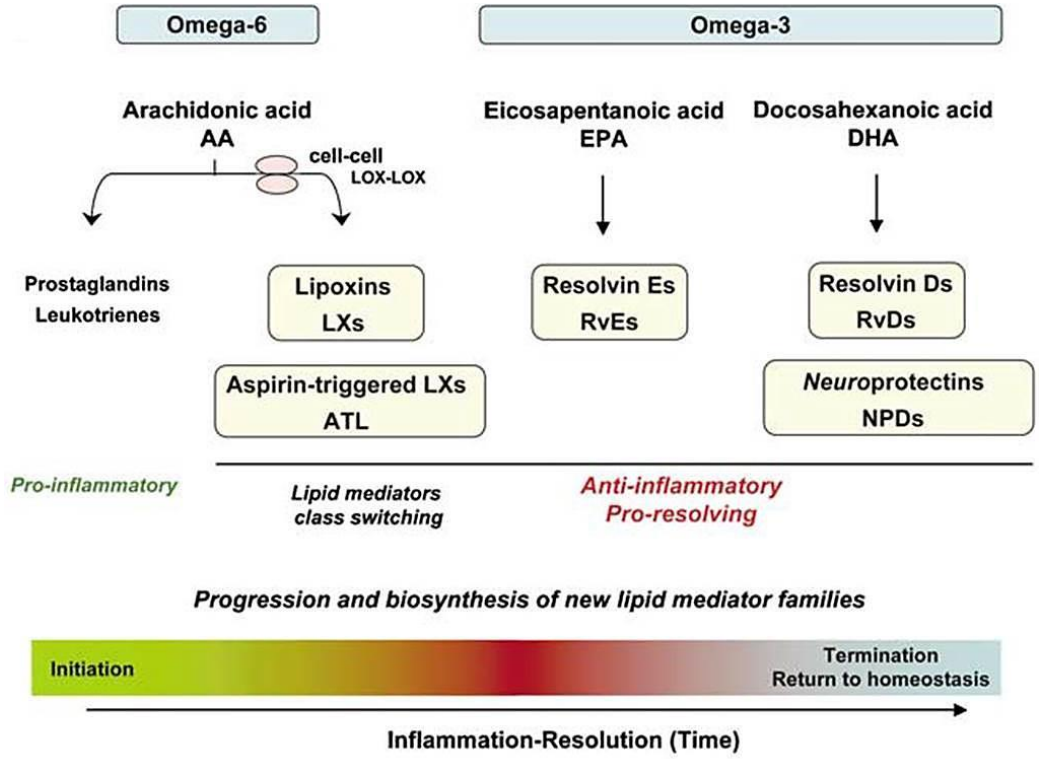

Figura 8. Mediadores e sinais químicos especializados são programados, em nível tecidual, para participar ativamente na resposta dos leucócitos requeridos para a resolução. Prostaglandinas e leucotrienos estão envolvidos no ínicio da inflamação. Com o tempo, ocorre uma mudança de classe em direção a mediadores lipídicos pró-resolução. Estes mediadores incluem o ω-6 (ácido araquidônico: AA), derivado de lipoxinas (LXs) e lipoxinas acionadas por aspirina (ATL), além da série ω-3 de ácido eicosapentaenoico (EPA) - resolvinas E (RvEs), de ácido docosahexaenoico (DHA) – resolvinas D (RvDs), e de neuroprotectinas (geradas em tecidos neurais). Extraído de Serhan e Chiang (2008).

O perfil dos PUFAs na dieta e o balanço entre os ácidos graxos n-3 e n-6 pode alterar os fosfolipídeos das membranas biológicas e conferir mudanças à fluidez da membrana celular (Abbot et al., 2012). Além da atuação nos processos inflamatórios, existem várias evidências do papel do ácido docosahexaenoico (DHA) na sinalização celular e na expressão gênica (Teitelbaum, Allan Walker, 2001; Akiyama et al., 2013). É sabido que o DHA pode modular e regular as vias do AR nas células prostáticas LNCaP, inibindo a proliferação celular mediada por andrógeno, interferindo negativamente nas atividades de transativação do AR (Chung et al., 2001) e promovendo degradação desse receptor (Hu et al., 2015). Em outro estudo *in vitro*, o crescimento de células humanas prostáticas estromais da linhagem WPMY-1 e da linhagem epitelial RWPE-1 foi inibido pelo DHA. Essa inibição ocorreu devido à expressão diminuída de ERα (receptor de estrógeno do tipo α) e AR e do bloqueio do ciclo celular nas fases G2/M, após exposição ao DHA (Wang et al., 2016). Estudos também mostram que o DHA é citotóxico para várias células cancerígenas, por sua habilidade de induzir a apoptose (Calviello et al., 1998; Zand et al., 2007). Em células da linhagem hematopoietica a ação pró-apoptótica do DHA é mediada pelo PPARγ (receptor de ativação da proliferação dos peroxissomos do tipo γ) que, por sua vez, regula positivamente o gene supressor tumoral p53 (Zand et al., 2007).

Estudos recentes apontam a importância do consumo de DHA, durante a gestação, para o melhor desenvolvimento do sistema nervoso central e, consequentemente, para a melhora da atenção e da memória em crianças pré-escolares (Ozias et al., 2007; Colombo et al., 2016; Ramakrishnan et al., 2016). Outro efeito positivo da suplementação da dieta materna com DHA é a redução dos nascimentos precoces (nascimentos antes da 34^{a} semana de gestação) (Yelland et al., 2016). Um acréscimo rápido de DHA ocorre durante o terceiro trimestre da gestação humana (Clandinin et al., 1980). Nos ratos, que são mais imaturos ao nascimento, o acúmulo desse lipídeo ocorre de forma pronunciada três dias antes do nascimento e continua durante o período de lactação (Green, Yavin, 1996).

Estudos prévios também têm apontado a importância do DHA para o sistema reprodutor, principalmente para a espermatogênese e para a qualidade espermática (Colon et al., 1986; Rejraji et al., 2006; Roqueta-Rivera et al., 2010). Uma proporção equilibrada de PUFAs n-3/n-6 na dieta é benéfica para a capacidade reprodutiva de mamíferos e aves (Yan et al., 2013; Feng et al., 2015). Ratos alimentados com dieta rica em PUFAs n-3/n-6 tiveram maiores níveis de testosterona, maior ganho de peso e menores taxas de deformidade espermática em comparação ao grupo controle (Yan et al., 2013). Resultados similares foram

observados por Feng et al. (2015) para o galo doméstico, depois do consumo de dieta suplementada com diferentes razões entre PUFAs n-3/n-6. Os PUFAs aumentaram a concentração de GnRH (hormônio liberador de gonadotrofina), FSH (hormônio folículo estimulante), LH e testosterona no plasma. Esse resultado foi positivamente relacionado à melhora na qualidade e na motilidade dos espermatozoides (Feng et al., 2015). O DHA sozinho também tem sido relacionado a concentrações aumentadas de testosterona no plasma sanguíneo. Búfalos machos alimentados com dieta rica em DHA tiveram níveis plasmáticos elevados de IGF-1 (fator de crescimento semelhante à insulina tipo 1) e andrógenos, tal como uma maior quantidade de DHA nos espermatozoides, o que contribuiu para aumentar a fluidez da membrana plasmática e elevar a qualidade dos gametas masculinos (Tran et al., 2016). Entretanto, a escassez de conhecimento sobre a atuação desse PUFA no desenvolvimento testicular, juntamente com a recomendação mundial de suplementação materna com DHA, torna imperativo o estudo de seus efeitos durante o desenvolvimento testicular.

I. 5. Os Receptores Nucleares

Como já mencionado, os desreguladores endócrinos com potencial antiandrogênico, tais como os metabólitos do DEHP e o DBP, interferem nos mecanismos de desenvolvimento dos gonócitos – proliferação, morte e diferenciação dessas células em espermatogônias (Chauvigné et al., 2009; Mylchreest et al., 1999). Recentemente, Rouiller-Fabre et al. (2015) propuseram que tais interferências podem ocorrer via receptores nucleares (NRs), presentes tanto nas células somáticas como nas células germinativas do testículo fetal e neonatal (Figura 9). Dentre esses receptores destacam-se o receptor de ativação da proliferação dos peroxissomos (PPAR) e o receptor X do fígado (LXR) (Rouiller-Fabre et al., 2015). Os PPARs são ligantes de uma grande variedade de ácidos lipofílicos naturais e sintéticos, como os ácidos graxos essenciais (Grygiel-Górniak, 2014). Esses NRs foram inicialmente descritos no tecido adiposo, onde controlam o armazenamento e o metabolismo de lipídeos, e posteriormente foram reportados como reguladores dos metabolismo, proliferação e diferenciação celular em outros tecidos (Theocharis et al., 2004; Rogenhofer et al., 2012; Strand et al., 2012; Grygiel-Górniak, 2014). O MEHP, mas não o DEHP, é ligante do PPAR γ (Desvergne et al., 2009) e medeia seus efeitos nas células. A avaliação dos efeitos da exposição ao MEHP sobre a expressão de mRNA de 48 diferentes NRs revelou que a maioria deles não variou no testículo fetal humano. Entretanto, o PPARγ e o NGFIB (fator de

crescimento do nervo do tipo IB) diminuíram, enquanto o LXRα aumentou (Muczynsky et al., 2012b). Além disso, usando citometria de separação de células com marcadores específicos para células germinativas e para células somáticas, os autores demostraram que o aumento da expressão de LXRα foi restrito às células somáticas e relacionado a um aumento da expressão de sete LXRα *downstream genes* envolvidos na síntese de lipídeos e de colesterol, bem como da proteína de ligação a elemento regulador de esterol (SREBP). Contudo, foi descrito que o DHA desempenha um papel oposto, diminuindo a expressão do fator de transcrição SREBP-1 e de enzimas envolvidas na síntese de lipídeos (Georgiadi, Kersten, 2012). Tais dados indicam que a ativação do metabolismo de lipídeos via LXRα é um efeito da exposição do testículo ao MEHP e a diminuição da expressão de PPARγ provavelmente está relacionada à redução no número de gonócitos (Muczynsky et al., 2012b). Sendo assim, levantamos a hipótese de que o DHA pode antagonizar com os efeitos adversos do MEHP durante o desenvolvimento testicular.

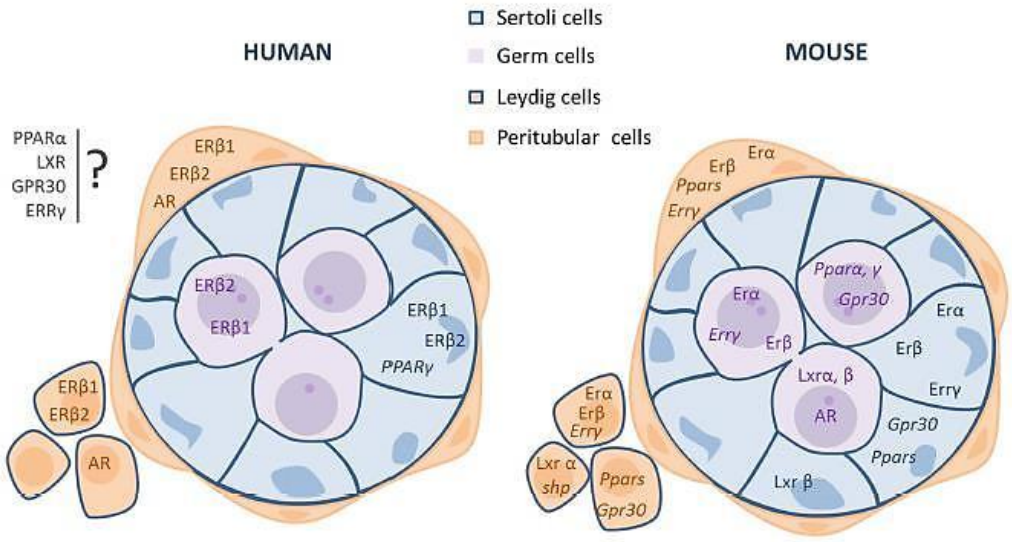

Figura 9. Localização dos receptores envolvidos nos efeitos dos desreguladores endócrinos nos testículos fetal e neonatal de humanos (esquerda) e de roedores (direita). A expressão do RNAm (itálico) e da proteína (romano) está indicada em cada tipo de célula: células germinativas, células de Sertoli e células do interstício (Leydig e peritubulares). Extraído de Rouiller-Fabre et al. (2015).

II. OBJETIVOS

Os objetivos do presente trabalho foram (1) avaliar se a exposição materna ao ftalato interfere no desenvolvimento neonatal do testículo do gerbilo da Mongólia, e (2) examinar, também, o efeito direto do DHA através do sistema de cultura organotípica, sobre o desenvolvimento do testículo fetal de camundongo, verificando se o DHA antagonizou com os efeitos sabidamente adversos da exposição materna aos ftalatos.

III. RESULTADOS

Os resultados do presente trabalho foram divididos na forma de dois manuscritos. O primeiro deles foi submetido à "*Reproduction, Fertility and Development*". O segundo encontra-se em fase de elaboração.

IV. MANUSCRITO 1

Effects of Gestational Exposure to Di-n-butyl Phthalate and Mineral Oil on Testis Development of the Mongolian Gerbil (*Meriones unguiculatus*)

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Short title: DBP and oil exposure on testis development

ABSTRACT

Phthalate esters, such as di-n-butyl phthalate (DBP) are endocrine disrupters that can affect the development of the testis in a species-specific manner. However, its interference in the male gonads of the Mongolian gerbil is unknown. The objective of this study was to evaluate whether maternal exposure to DBP interferes with the development of the gerbil testis during the first six weeks of life. For this purpose, the testes of animals born from control mothers or mothers exposed to mineral oil or to DBP (100 mg/kg from 8 to 23 days postconception) were evaluated at ages of 1, 7, 14, 28, 35 and 42 days. Our results indicate that DBP did not change androgen levels at birth but affected steroidogenic activity at the end of the first week. It also impaired the number of germ cells at 7 days. Deleterious effect of mineral oil on steroid hormones synthesis was also observed. However, since AR expression was not modified by exposure to DBP, changes in androgen levels probably occurred by pathways independent of androgen receptors.

1. INTRODUCTION

Phthalate esters are endocrine disrupters (EDs) that can affect germ cells and somatic cells of the testis and also testosterone production (Chauvigné et al., 2009; Svechnikov et al., 2010). These compounds, such as di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP), are common chemical additives used in the manufacture of plastic products and are found in toys, cosmetics, food packaging and medical equipment (Kavlock et al., 2002; Habert et al., 2014).

Concerning exposure to DBP during the embryonic period, Mylchreest et al. (1999) observed an increased proliferation of Leydig cells, the presence of multinucleated gonocytes within the seminiferous cords and decreased testosterone levels in the male fetal rat. However, their interference seems to be species-specific. Comparing the effects of different EDs on the development of fetal human, rat and mouse testes, Habert et al. (2014) concluded that the anti-androgenic effect of phthalates, detected in the rat testis, does not exist in the human species. While mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite of DEHP, impairs the development of germ cells in the fetal human (Lambrot et al., 2009) and mouse testes (Lehraiki et al., 2009) without altering testosterone production, gestational exposure of rats to DBP causes a reduction in androgen levels (Hallmark et al., 2007).

Gonocytes, germ cells present in the fetal and neonatal testis, are the precursors of spermatogonial stem cells (SSCs) and type A spermatogonia of the first spermatogenic wave (Manku and Culty, 2015; Yoshida et al., 2006). The processes of proliferation, death and differentiation of these cells are essential for the proper establishment of spermatogenesis (Culty, 2009; Manku et al., 2016).

Considering that the action of phthalates on the male reproductive system points to a wide species-, age-, and dose-specific variation in the interference of these compounds, mainly in serum testosterone levels and in the number of germ cells (Parks et al., 2000; Hallmark, et al., 2007; Culty et al., 2008; Lambrot et al., 2009; Lehraiki et al., 2009), we decided to use an alternative animal model, the Mongolian gerbil (*Meriones unguiculatus*), to evaluate the consequences of prenatal exposure to di-n-butyl phthalate on the male gonad. Recent reports from our laboratory have characterized the testicular development of this rodent, including the sperm parameters (Pinto-Fochi et al., 2014), and also indicated that the gerbil is an excellent model to assess the consequences of EDs in prostatic lesions (Perez et al., 2012; de Jesus et al., 2015).

Thus, the present study evaluated the consequences of gestational exposure to DBP on the testis development of the Mongolian gerbil from 1 to 42 days of age with emphasis on germ cell differentiation and steroidogenesis.

2. MATERIAL AND METHODS

2.1. Animals and experimental design

Male and female Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Animal Breeding Center of São Paulo State University - UNESP, Institute of Biosciences, Humanities and Exact Sciences – IBILCE. The animals were kept in the Animal Breeding Center of the same institute, at a controlled temperature (23-25°C), humidity (40-60%), under 12h light/dark cycle, and treated with filtered water *ad libitum*. The experimentation procedures were performed in accordance with the guidelines of the Ethics Committee on Animal Experimentation (CEUA/UNESP, protocol 089/2013).

Three groups of male gerbils at the following ages were used: 1, 7, 14 (n = 12 per group), 28, 35 and 42 (n = 6 per group) postnatal days. They were exposed to 100 mg/kg/day of DBP (Sigma Chemical Co., St Louis, MO, USA) diluted in 0.1 mL of mineral oil, via maternal gavage, from gestational day 8 to 23 (DBP group), to the mineral oil only (O group) or no treatment (C group). This dose was chosen based on the extensive dose-response study of Mylchreest et al. (2000).

A total of 90 families were used, comprising 90 males (14 weeks old) and 90 females (12 weeks old). The females were checked for the presence of spermatozoa by vaginal smear and, a positive finding being considered day 0 of gestation. The birth date of pups was defined as day 1 and the identification of the sex was based on anogenital distance, which was measured with a digital caliper (King Tools, digital caliper, 0-150mm).

The gerbils were killed by deepening anesthesia, followed by decapitation for the purpose of blood collection. Testes were dissected and weighed, and the gonadosomatic index (GSI) was obtained from the formula GSI = (Testicular Weight/Body Weight) x 100.

2.2. Testis processing for light microscopy

Testes were fixed by immersion for 6 h (1, 7 and 14 days) or 12 h (28, 35 and 42 days) in Bouin fluid, washed in 70% alcohol for the removal of picric acid and processed for inclusion in Paraplast (Histosec, Merck, Darmstadt, Germany) or historesin (Leica-historesin embedding kit, Nussloch, Germany). The right testis was included in Paraplast and the left

one in historesin (n = 6 per group), in the case of the gerbils at 1, 7 and 14 days. For the oldest age groups (28, 35 and 42 days), half of the right testis was included in Paraplast and half in historesin. After HE staining of the historesin sections (3µm thick), the diameters of 20 seminiferous cords or tubules were determined, per animal (n = 5 per group), at 20x lens, using the Image Pro-Plus software (Media Cybernetics version 4.5, MD, USA).

The digital images were obtained with an Olympus BX60 photomicroscope coupled with the Scanner System and Image Analyzer (Image Pro Plus® Media Cybernetics), and the BX61VS camera (Olympus Corporation, Tokyo, Japan) attached to the VS120-S5 scanner from the same manufacturer.

2.3. Immunocytochemistry

Immunocytochemical reactions (using Paraplast sections of 5µm) were performed for anti-Müllerian hormone (AMH) and androgen receptor (AR). Briefly, paraffin sections were immersed in citrate buffer (pH 6) at 92°C, for 45 min, for the purpose of antigen retrieval. The blocking of endogenous peroxidase was achieved by incubation with 3% H_2O_2 in methanol, for 20 min. Then, the tissue sections were incubated with 5% non-fat milk in PBS, for 30 min, to block non-specific protein-linkage. Incubations with primary antibodies were performed overnight at 4°C in 1% BSA using the following antibodies and dilutions: 1:75 rabbit IgG anti-human AR (sc-816, Santa Cruz Biotechnology, CA, USA) and 1:100 goat IgG anti-human AMH (sc-6886, Santa Cruz Biotechnology, CA, USA).

The above-mentioned reactions were then incubated with the biotinylated secondary antibody, followed by the ABC avidin-biotin complex Kit (Santa Cruz Biotechnology), for 45 min, at 37°C. Sections were revealed with diaminobenzidine (DAB) for approximately one minute, and counterstained with Hematoxylin. The negative control was obtained by omission of the primary antibody.

2.4. Numerical density and total number of gonocytes

The numerical density (Nv) of gonocytes, or the number of these cells in a given tissue volume, was estimated for the seminiferous cord of the gerbils at 1, 7 and 14 days. This parameter was estimated in order to examine whether the number of gonocytes, in the same volume of seminiferous cords, varied among the different experimental groups. These analyses were conducted based on the procedures of Rosen-Runge and Leik (1968) and Zogbi et al. (2012). For this purpose, the testicular volume (mm^3) was determined, being considered

equal to fresh testis weight (mg) without correction for density. The tissue sections were previously submitted to immunocytochemistry for AMH, and gonocytes appeared unmarked against the AMH-positive cytoplasm of the Sertoli cells. For each animal we used two different histological sections and 10 random fields per section, examined with a 20x lens. After application of the M132 reticle, the volume density was determined by the percentage of dots which covered the seminiferous cords. Based on seminiferous cord volume density the absolute volume of cords was calculated for each organ. The crude counting (CC) of gonocytes was then estimated using five histological sections, from approximately equidistant regions of the testis, for each animal. For the purpose of estimating CC only the gonocytes with evident nuclei were counted. The Nv was calculated as the ratio between CC and seminiferous cord volume.

Considering the density of the testis to be equal to 1, testicular volume was equivalent to mass. Thus, the total number of gonocytes (NG) was obtained by multiplying the weight of the gonad by the Nv, which corresponds to the number of gonocytes per mm^3.

2.5. Density of relocated gonocytes and of mitotic figures

The densities of relocated gonocytes and mitotic cells in the seminiferous epithelium were determined for gerbils at 1, 7 and 14 days. We used five different histological sections per testis, for each animal, subjected to immunocytochemistry for AMH, which were analyzed with a 40x lens throughout their length. For each tissue section the cells with typical gonocyte morphology, in direct contact with the basement membrane (relocated germ cells) were counted, using the Image Pro-Plus software (Media Cybernetics version 4.5, MD, USA). The migratory event of the germ cells from the center to the periphery of the neonatal seminiferous cords, also known as relocation, is indispensable for the differentiation of the gonocytes into spermatogonia (Roosen-Runge and Leik, 1968; Clark and Eddy, 1975; McGuiness and Orth, 1992).

The cell proliferation in the seminiferous epithelium was also estimated for gerbils at 1, 7 and 14 days using five histological sections per testis, for five different pups, after staining for AMH. For each section the cell proliferation was estimated by counting the density of mitotic figures (cells from metaphase to telophase). The values obtained were expressed as the number of mitotic figures/mm^3 of seminiferous cord, using the absolute volume of cords previously determined for the gonocyte count.

2.6. Estimation of apoptosis index

The apoptotic cells were detected using the DNA fragmentation assay associated with cell death, based on the TUNEL reaction, according to the kit instructions (ApopTag Plus in situ, Apoptosis Detection Kit, Millipore, 57101, CA, USA). Briefly, after digestion with proteinase, the histological sections were submitted to procedures designed to inactivate endogenous peroxidase (3% H^2O^2 in PBS, for 5 min), and then incubated with the enzyme deoxynucleotidyl terminal transferase (TdT) for 1 h, at 37°C. At the end of the reaction, the sections were incubated with peroxidase, revealed with diaminobenzidine (DAB) and counterstained with Hematoxylin. The estimation of apoptosis in the seminiferous epithelium was made for five animals in groups from 1 to 14 days. The total area of the histological section was estimated from images scanned images at 40x magnification, using the above program, excluding the region corresponding to the tunica albuginea. The total number of apoptotic cells per histological section was divided by the corresponding area.

2.7. Testicular maturation

The grade of testicular maturation was evaluated for the age of 28 days. First the analysis of the number of seminiferous cords with or without light was carried out, considering that the presence of light and testicular fluid reflects the beginning of Sertoli cell differentiation. A total of 200 cords per testis (n = 5 per group) was scanned from a continuous area of the histological section at 400x magnification.

The beginning of spermatogenesis was also evaluated. One testis from each animal (n = 5 per group) was scanned under a microscope and the seminiferous cords staged according to the most advanced cell type in the spermatogenic lineage, as follows: A spermatogonia (A); B spermatogonia (B); leptotene primary spermatocytes (L), zygotene primary spermatocytes (Z) and pachytene primary spermatocytes (P). These cells were identified as described by Clermont and Perey (1957), with the 20x lens, in histological sections stained with HE. At least 150 tubules were evaluated for each animal and the data were expressed as percentages of tubules, according to the most advanced cell type present.

2.8. Western blotting

Both testes of six animals at 1, 7 and 14 days and the left testes of the animals at 28, 35 and 42 days were frozen in liquid nitrogen and kept at -80°C in order to evaluate 17β-hydroxysteroid dehydrogenase (17β-HSD), androgen receptor (AR) and anti-Müllerian

hormone (AMH) expression, by Western blotting. Total extracts were obtained after testis homogenization in RIPA buffer (Sigma, MO, USA) and centrifugation. The protein concentrations of supernatants were quantified by the Bradford method (Bradford, 1976), and 30 mg of protein was subjected to SDS-PAGE. After electrophoresis, proteins were transblotted onto a nitrocellulose membrane (GE Healthcare). The blot was blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 30 min, incubated overnight at 4°C with 1% BSA in TBST containing a 1:300 dilution of the primary antibodies (Santa Cruz Biotechnology, CA, USA: 17β-HSD - sc-32872; AR - sc-816; MIS - sc-6886) or a 1:1000 dilution of anti-actin β (sc-47778; Santa Cruz Biotechnology, CA, USA), washed (3 × 10 min) in TBST, and incubated for 1h at 4°C with secondary antibody, followed by three washes (10 min) in TBST. Antibody detection was revealed using the ELC chemiluminescent detection kit (GE Healthcare). Protein expression was normalized to the actin β values. The density of immunolabeled bands was analyzed using the Image J 1.34 (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) densitometry program.

2.9. Hormone dosage

Testosterone and estradiol dosages were performed using ELISA: Cayman Chemical Company, Ann Arbor, MI, USA - Item # No. 582701 (testosterone) and Item # No. 582251 (estradiol), according to the manufacturer's specifications. The readings were performed on an Epoch™ Multi-Volume Spectrophotometer System (BioTekInstruments, VT, USA) reader.

2.10. Statistical analyses

Statistical analyses were performed with GraphPad 5.0 (© 1992-2007 GraphPad Software, Inc.) by One-Way ANOVA test. Data are presented as individual values; bars indicate mean and SEM. $p < 0.05$ was considered significantly different.

3. RESULTS

3.1. DBP increases the total number of gonocytes at 7 days

Prenatal exposure to DBP resulted in increased pup weight at birth compared to the control group (Fig. 1A). An interference of phthalate in the number of germ cells was also observed. At the end of the first postnatal week, the NG of the DBP group was lower than that of the control group (Fig. 2A). However, the numerical density, which refers to the number of

germ cells per mm^3 of testis was unaffected (Fig 2B) and no interference of the di-n-butyl phthalate was observed in the relocation process of the germ cells from 1 to 14 days (Fig. 2C-D, G).

In addition to decreasing the number of gonocytes, the proliferation of cells within the seminiferous cords (germ cells and Sertoli cells) was also affected by di-n-butyl phthalate (Fig. 3A-D). While the number of mitotic figures was lower at birth, in the gerbils at 7 days the proliferation increased in the DBP groups compared to the control (Fig. 3A-B, D). Prenatal exposure to phthalate also resulted in the appearance of multinucleated gonocytes (MNG) at the age of 7 days (Fig. 3E).

With regard to cell death, there was a linear increase in the frequency of apoptotic cells from 1 to 14 days (Fig. 3G-K) but no statistical difference was observed among groups in any of the three ages evaluated. Another parameter unaffected by DBP exposure was the anogenital distance at birth (control = 0.859 ± 0.062 mm, oil = 0.085 ± 0.064 mm, DBP = 0.085 ± 0.056 mm).

Prenatal exposure to di-n-butyl phthalate also resulted in testicular changes at the end of the fourth week of life. Seminiferous tubule staging indicated that, at 28 days, the frequency of tubules whose most advanced germ cells were primary spermatocytes in pachytene was higher in relation to those with spermatogonia A and B (Fig. 5A-F). Considering only the tubules at primary spermatocyte in leptotene and zygotene, DBP treatment resulted in an increase of the first by 63% and a decrease of the later by 49% in comparison with the control groups (Fig. 5A).

3.2. Mineral oil changes testicular development during the first six weeks of postnatal life

In addition to the effects of DBP on the NG of gonocyte, mineral oil alone was responsible for changes in the testicular development of the Mongolian gerbil. At the end of the first week of life, the animals whose mothers had been exposed to the oil had a lower testicular weight (Fig. 1B) and a decreased number of germ cells (Fig. 2B), in addition to an increase in germ cell and Sertoli cell proliferation (Fig. 3A-C) compared to the respective control groups. Interference from the oil was also observed in the second week of life. At 14 days testicular weight (Fig. 1B) and GSI (control = 0.882 ± 0.017; oil = 0.775 ± 0.047; DBP = 0.902 ± 0.029) were decreased after gestational exposure to oil. Also at this age the relocated

gonocyte density was higher in the O group when compared to the control group (Fig. 2C-F, H).

Prenatal exposure to mineral oil also affected the percentage of seminiferous cords and interstitial tissue, as well as the diameter of seminiferous cords/tubules. Pups at the age of 1 day belonging to the O group presented a lower percentage of cords than those of interstitial tissue (Fig. 4A), while gerbils at 42 days also from the group exposed to mineral oil had seminiferous tubules larger than those from the control groups (Fig. 4B).

At 28 days, the oil probably led to a decrease in the percentage of primary spermatocytes in zygotene in relation to the control group (Fig. 5A) and to a lower percentage of seminiferous cords with lumen, at the same age, in comparison with the DBP group (Fig. 5G).

3.3. Both DBP and mineral oil alter steroidogenesis

The general analysis of testosterone levels showed a reduction of this hormone at the age of 7 days. The concentration of testosterone was not significantly different at birth after exposure to di-n-butyl phthalate in comparison with the non-exposed pups. However, at the end of the first week of life, androgen levels were higher in both O and DBP groups when compared to the controls (Fig. 6A).

Estrogen plasma levels were lower at birth and higher at the end of the fifth week in the animals belonging to the DBP group in relation to the control groups (Fig. 6B). At 28 days, the animals whose mothers had only received the oil had estradiol levels approximately 53% higher than those of the same age in the control group (Fig. 6B).

3.4. Prenatal exposure to DBP does not alter the function of Leydig cells in the prepubertal period

The effect of gestational exposure to DBP on Leydig cells was assessed by AR and 17β-HSD expression. No difference in the intensity of AR labeling was observed among groups from 1 to 14 days (Fig. 7). However, in contrast with observations in histological sections from gerbils at 1 and 14 days of age (Fig. 7A-C, H-J), at 7 days, the gonocytes presented cytoplasmatic staining for AR (Fig. 7D-G). Immunocytochemical detection of 17β-HSD also revealed no obvious change in the labeling intensity of Leydig cells among groups at 42 days (Fig. 8A-D). The same was observed for the content of 17β-HSD in testicular lysates of gerbils at this age (Fig. 8E-F).

4. DISCUSSION

It is known that phthalate esters act as endocrine disrupters and affect testicular development due to disturbances in the action/signaling of androgens and estrogens (Mylchreest et al., 1999; Chauvigné et al., 2009; Chen et al., 2013). However, the consequences on testis development after gestational exposure to DBP had not previously been examined for the Mongolian gerbil. Our results showed a greater interference of DBP and mineral oil in gonadal development and on the concentration of testosterone and estrogen during the first week of postnatal life.

One of the most common deleterious effects of phthalates on testicular development is the decrease in the number of germ cells in both fetal and neonatal periods (Ferrara et al., 2006; Lambrot et al., 2009; Lehraiki et al., 2009). Our results demonstrated that exposure to DBP resulted in a decreased number of germ cells at 7 postnatal days. Ferrara et al. (2006) observed a reduction in the number of gonocytes by 37%, 53%, 79% and 80% at 21.5 days post conception (dpc) and on postnatal days 4, 8 and 15, respectively, after exposure of female parent rats to DBP. The decrease in the number of germ cells at 4 and 8 days was related to a 28% drop in the gonocyte proliferation index at 6 days. In the case of gerbils, the lower density of mitotic figures at birth in the DBP group was the probable cause of the reduction of 28% in the NG at 7 days compared to control of same age. It is important to note that, in the work of Ferrara et al. (2006), the reduction in the number of germ cells occurred in the fetal (21.5 dpc) and neonatal periods (4, 8 and 15 postnatal days), with a later recovery of the testes from 25 to 90 days of age. In another experiment, exposure of co-cultures of Sertoli cells and neonatal gonocytes from rats at 2 days to 1mM of MEHP caused the detachment of gonocytes from the monolayers of Sertoli cells after 12h, and many gonocytes were lost with the exchange of the culture medium after 24h. Sertoli cell proliferation was also suppressed (Li et al., 1998). All these results demonstrate that the neonatal testis is particularly sensitive to exposure to phthalate esters.

At 14 days most gonocytes were attached to the basement membrane and only at this age was any difference observed in the relocation of these cells. Exposure to mineral oil increased the density of relocated gonocyte by 36% compared to the control. The migratory behavior of this cell type during the neonatal period is fundamental for the resumption of mitosis and for its differentiation in spermatogonia (Roosen-Runge and Leik, 1968; McGuinness and Orth, 1992; Tres and Kierszenbaum, 2005). It was also observed that, while the numerical density of gonocytes decreased exponentially over the first week due to their

transition to spermatogonia, the number of apoptotic cells increased, following the pattern previously described for this species (Pinto et al., 2010). However, there was no statistical difference between groups from 1 to 14 days for the frequency of apoptotic cells. Moody et al. (2013) also found that altered apoptosis is not a significant consequence of prepubertal exposure to DBP. After treatment with 500 mg/kg of DBP for 3 days (from 4 to 7 postnatal days), neither cleaved caspase-3-positive cells nor TUNEL-positive cells differed between treated and control groups.

As observed for the rat, which exhibits a testosterone peak a few hours after birth (Corbier et al., 1992), the hormonal dosage indicated higher levels of this hormone in the gerbils at birth compared to the animals at 7 and 14 days. Although testosterone levels did not vary between groups at 1, 14, 28, 35 and 42 postnatal days, DBP exposure minimized the drop in circulating testosterone at 7 days. In an experiment conducted by Lehraiki et al. (2009) the exposure of mice fetuses at 13.5, 15.5 and 18.5 dpc to 20 or 200μM of MEHP (kept for 1 to 3 days in culture) revealed that changes in steroidogenesis were highly complex. Exposure to phthalate stimulated, inhibited or had no effect on testosterone production depending on the age and concentration of MEHP. In the rat, the main deleterious effect of phthalate esters is their antiandrogenic action (Parks et al., 2000; Hallmark et al., 2007; Culty et al., 2008). In humans, treatment with MEHP (10^{-4}M) did not interfere with basal or LH-stimulated testosterone production in fetal testis culture (Lambrot et al., 2009). Thus, the effects of phthalates on steroidogenesis are largely species-specific (Habert et al., 2014).

Regarding the function of Leydig cells, prenatal exposure of the Mongolian gerbil to 100 mg/kg of DBP did not alter the levels of 17β-HSD at the end of the sixth week, a fact also observed by Motohashi et al. (2016) after gestational exposure of rats at the same dose of DBP used in the present study but from 12 to 21 dpc. In the fifth and seventh weeks the mRNA and the protein levels for 3β-HSD, P450c17 and 17β-HSD were similar in the control and DBP groups (Motohashi et al., 2016).

In addition to the antiandrogenic effect, the estrogenic effect of DBP has already been confirmed for the rat *in vitro* and *in vivo* (Chen et al., 2013; Li et al., 2014). Bao et al. (2011) observed an increase in estrogen levels in pubertal rats (approximately 4 weeks old) exposed to DBP (500 mg/kg for 30 days). However, at a concentration of 100 mg/kg/day, the same as that used in our study, there was no difference between treated and control animals (Bao et al., 2011). According to our results, prenatal expose to DBP resulted in lower levels of this hormone at birth and in higher levels at 35 days compared to the controls at the same age.

Mineral oil was chosen as a vehicle due to previous studies results. They indicate that vegetable oils contain phytoestrogens that can affect testicular differentiation and other orgnas regulated by steroid hormones (Rodriguez-Gomez et al., 2014; Seliukova et al., 2014). However, our results indicated that mineral oil itself has an effect on the development of the male gonads and on estrogen and testosterone levels in the gerbil. Cardoso et al. (2014) observed that not only mineral oil but also olive and sunflower oils (100 mg/kg for 10 days) reduced the acrosome integrity of the gametes. The *in vitro* penetration rate was also reduced for all vehicles. In addition to sperm damage, oily vehicles can also increase the toxicity of some chemicals (Yoshimura et al., 2003), cause deleterious effects on oocytes and impair embryonic survival (Otsuki et al., 2009). In the case of the Mongolian gerbil, maternal exposure to mineral oil resulted in a lower relative frequency of seminiferous cords at birth, in decreased testis weight and total number of gonocytes at the end of the first week of life and in increased estradiol levels at 28 days. These results clearly indicate an estrogenic effect of the oil in addition to its interference in testicular development.

The pattern of AR expression in the neonatal testis indicated by immunocytochemical analysis is in accordance with that described by Williams et al. (2001) for neonatal rats (1-12 days postpartum). The peritubular cells show an intense expression of this receptor, the Sertoli cells exhibit little or no expression and there is no expression at all in the gonocytes (Williams et al., 2001). However, in the case of the gerbil, a slight labeling of the gonocyte cytoplasm was observed at the age of 7 days alone, a fact also described by Pinto et al. (2010). Immunocytochemical analysis revealed no marked changes in the intensity or quantity of AR-labeled cells in the C, O or DBP groups. Thus, the changes in steroidogenesis observed at the end of the first week of life probably occurred independently of androgen receptors. According to Moody et al. (2013), although oral administration of DBP (from 4 to 14 postnatal days) affected androgen concentration at 14 days, no obvious difference in the expression and location of AR was observed between the group exposed to the xenobiotic and the control group. A previous study also emphasized that phthalate esters inhibit testosterone synthesis during fetal life but do not appear to be AR antagonists (Gray et al., 2001).

A decrease in anogenital distance is another common effect of exposure to phthalate esters and has been confirmed in both neonates (Parks et al., 2000) and prepubertal rodents (Moody et al, 2013), following maternal exposure to DEHP and DBP, respectively. This alteration was associated with the antiandrogenic activity of phthalates. Neither the anogenital distance nor the testosterone concentrations of the Mongolian gerbil at birth were found to be

any different in the C, O or DBP groups. However, the presence of multinucleated gonocytes was observed in the gerbil testis exposed to phthalate at 7 days. This fact was also noted *in vivo* by Parks et al. (2000) in rats of 20 dpc and 3 postnatal days, treated with DEHP during the prenatal period, and by Ferrara et al. (2006) in rat fetuses at 19 dpc after maternal gavage with DBP. Lehraiki et al. (2009) also observed MNG in mouse testes at 15.5 and 18.5 dpc in culture after exposure to MEHP. This did not occur in human fetal testis culture also exposed to MEHP, suggesting that rodents are more prone to this type of germ cell malformation than humans, at least during the fetal period

In summary, prenatal exposure to DBP did not change the anogenital distance or the number of gonocytes at birth but increased testosterone levels at the end of the first week of life by pathways independent of androgen receptors. Di-n-butyl phthalate also impaired the proliferation within the testicular cords at 1 day and the number of germ cells at 7 days. In addition to the effects of phthalate the mineral oil increased testosterone and estrogen synthesis at 7 and 28 days, respectively.

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LEGEND OF FIGURES

Figure 1. Body weight (A) and testicular weight (B) variation of gerbils from 1 to 14 days (n = 12) and from 28 to 42 days (n = 6) belonging to the control (C) groups or to exposed groups, in the prenatal period, to oil (O) or to di-n-butyl phthalate diluted in oil (DBP). Histograms represent the mean ± SEM. * $p < 0.05$ and ** $p < 0.001$.

Figure 2. (A) Total number (NG), (B) Numerical density (Nv) and (C) Density of relocated gonocytes of gerbils belonging to the control (C) groups or to groups exposed to oil (O) or to di-n-butyl phthalate diluted in oil (DBP), during gestation, at the ages of 1, 7 and 14 days. (D-G) Histological sections of gerbil testis at 14 days belonging to the C (D), O (F) or DBP groups, submitted to immunocytochemistry for anti-Müllerian hormone (AMH). The negative control is shown in E. (H) Western blotting analysis illustrates that the AMH antibody detected band at expected size (Molecular weight ~ 70-74 KDa, arrowhead) in gerbil testis lysates at 1 day; size marker are indicated on the left. Dark arrows point to relocated gonocytes. Bars = 20µm. Histograms represent the mean ± SEM. * $p < 0.05$.

Figure 3. (A) Density of mitotic figures. (B-E) Histological sections of gerbil testis at 7 days belonging to the control groups (B) or to the exposed groups, in the prenatal period, to oil (C) or to DBP (D-E), submitted to immunocytochemistry for anti-Müllerian hormone (AMH). The negative control is shown in 2E. (F) Frequency of apoptotic cells. (G-J) Histological sections of gerbil testis submitted to the TUNEL reaction at the ages of 1 (H), 7 (I) and 14 days (J). (G) Negative control. Dark arrows point to mitotic figures. Legend: Arrowheads – apoptotic cells; C - control group; DBP - group exposed to di-n-butyl phthalate diluted in oil during gestation; G – gonocytes; IT - interstitial tissue; O - group exposed to mineral oil during gestation; Red arrow - multinucleated gonocyte; S - Sertoli cells; SC - seminiferous cords. Bars: B, C, D, G, H, I, J and K = 20µm; E = 10µm. Histograms represent the mean ± SEM. * $p < 0.05$ and ** $p < 0.001$.

Figure 4. (A) Stereological data of the relative frequency of tissue compartments in the testis (seminiferous cords and interstitial tissue) in gerbils from 1 to 14 days belonging to the control group (C) or to groups exposed to oil (O) or to di-n-butyl phtalate (DBP). (B) Diameter of the seminiferous cords/tubules of gerbils from 1 to 42 days belonging to the C, O or DBP groups. Histograms represent the mean ± SEM. * $p < 0.05$ and *** $p < 0.0001$.

Figure 5. Analysis of the degree of sexual maturation of the control group (C) or of groups exposed to oil (O) or to di-n-butyl phthalate (DBP), during gestation, at the age of 28 days. (A) Percentage of seminiferous cords at each stage of the cycle, from spermatogonia A to spermatocyte in pachytene. (B-F) Seminiferous cords in various stages of differentiation. (G) Frequency of seminiferous cords with lumen in the C, O and DBP groups. Legend: A - spermatogonias A; B - spermatogonias B; L - primary spermatocytes in leptotene; P - primary spermatocytes in pachytene; Z – primary spermatocytes in zygotene. Bars = 10µm. Histograms represent the mean ± SEM. * $p < 0.05$ and *** $p < 0.0001$.

Figure 6. Testosterone (A) and Estrogen (B) plasma levels of control (C) or gerbils exposed to oil (O) or to di-n-butyl phthalate (DBP), during gestation, from 1 to 42 days. Histograms represent the mean ± SEM. * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

Figure 7. Histological sections of testis submitted to immunocytochemistry for androgen receptor (AR). The ages of the gerbils are shown on the left. (E) Negative control. Legend: C - control group; Dark arrows - unlabelled gonocytes; DBP - group exposed in the prenatal period to di-n-butyl phthalate diluted in oil; F - AR-negative nuclei of fetal Leydig cells; IT - interstitial tissue; Light arrows - AR-positive cytoplasm of gonocytes; O - group exposed in the prenatal period to mineral oil alone; P - AR-positive peritubular myoid cells; Red arrow-relocated gonocytes; S - unlabelled Sertoli cells; SC - seminiferous cords. Bars: A-I = 10µm; J = 20µm. (K) Western blotting analysis illustrates that the AR antibody detected band at expected size (Molecular weight ~ 110 KDa, arrowhead) in gerbil testis lysates at 1 day; size marker are indicated on the left.

Figure 8. (A-D) Testicular histological sections of gerbils belonging to the control (A), O (B) and DBP (C) groups at 42 days, submitted to immunocytochemistry for 17-beta-hydroxysteroid dehydrogenase (17β-HSD). (D) Negative control. Asterisks indicate the cytoplasmic labeling of Leydig cells. Bars = 10µm. (E) Western blotting analysis of the 17β-HSD content in the control testis or in testis exposed to oil or to DBP at 42 days. (F) Relative density of 17β-HSD normalized to actin β. Histogram represents the mean ± SEM. No statistical difference was observed among groups.

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Conflicts of Interest: The authors declare no conflicts of interest.

Figure 1

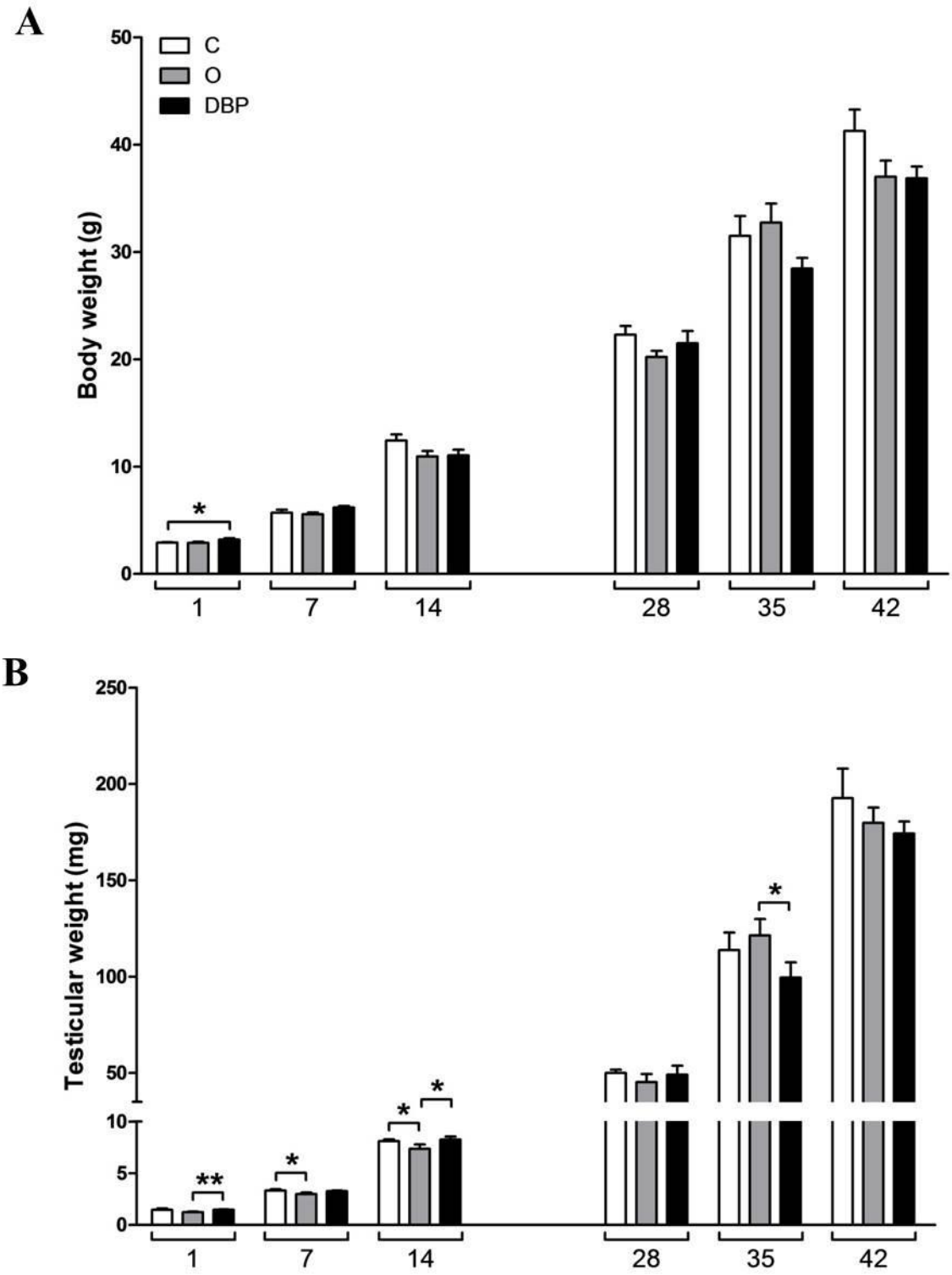

A
C
O
DBP
Body weight (g)
50
40
30
20
10
0
*
1
7
14
28
35
42
B
Testicular weight (mg)
250
200
150
100
50
10
5
0
**
*
*
*
*
1
7
14
28
35
42

Figure 2

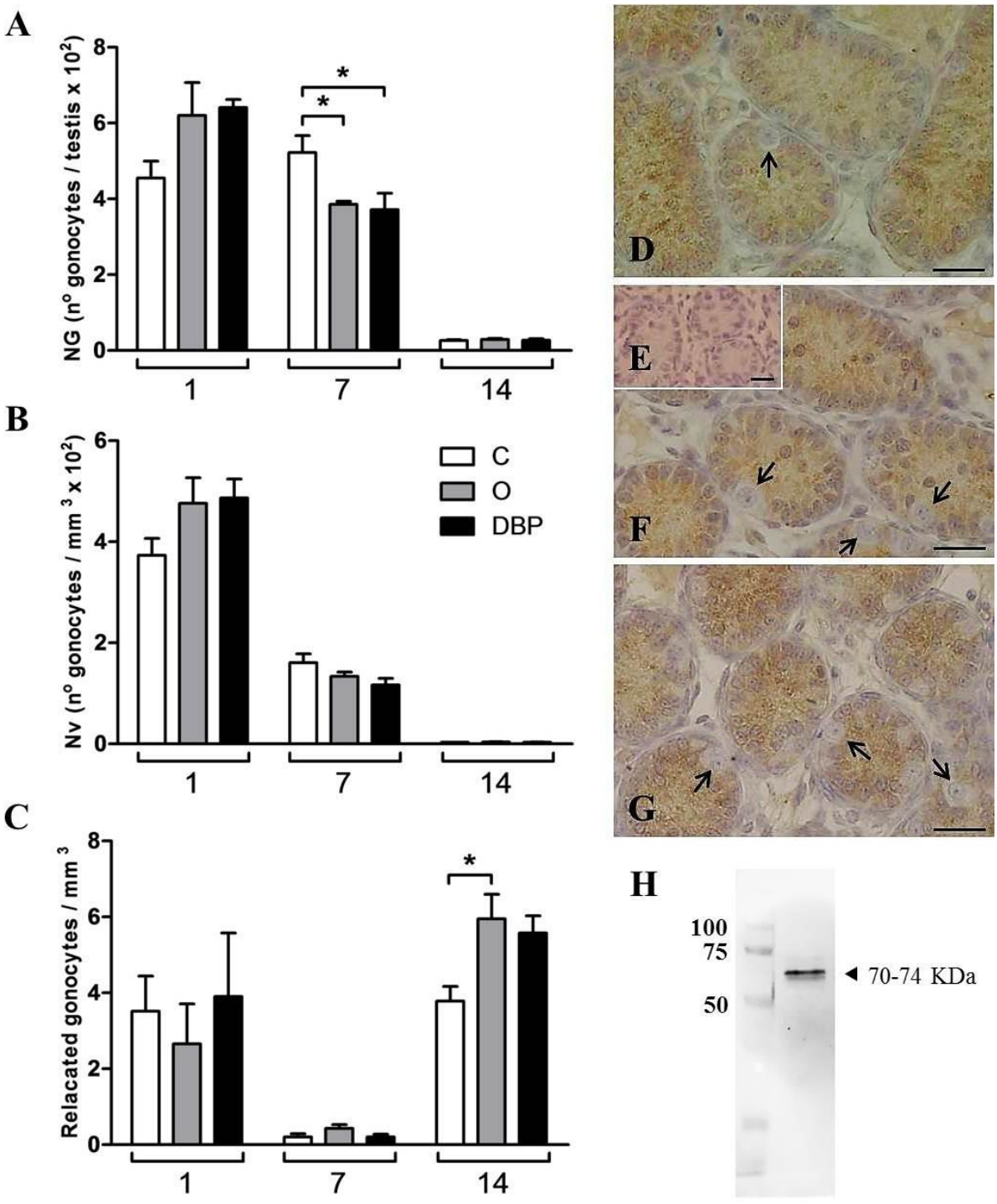

A
NG (nº gonocytes / testis x 10²)
8
6
4
2
0
*
*
1
7
14
B
Nv (nº gonocytes / mm ³ x 10²)
6
4
2
0
C
O
DBP
1
7
14
C
Relacated gonocytes / mm ³
8
6
4
2
0
*
1
7
14
D
E
F
G
H
100
75
50
70-74 KDa

Figure 3

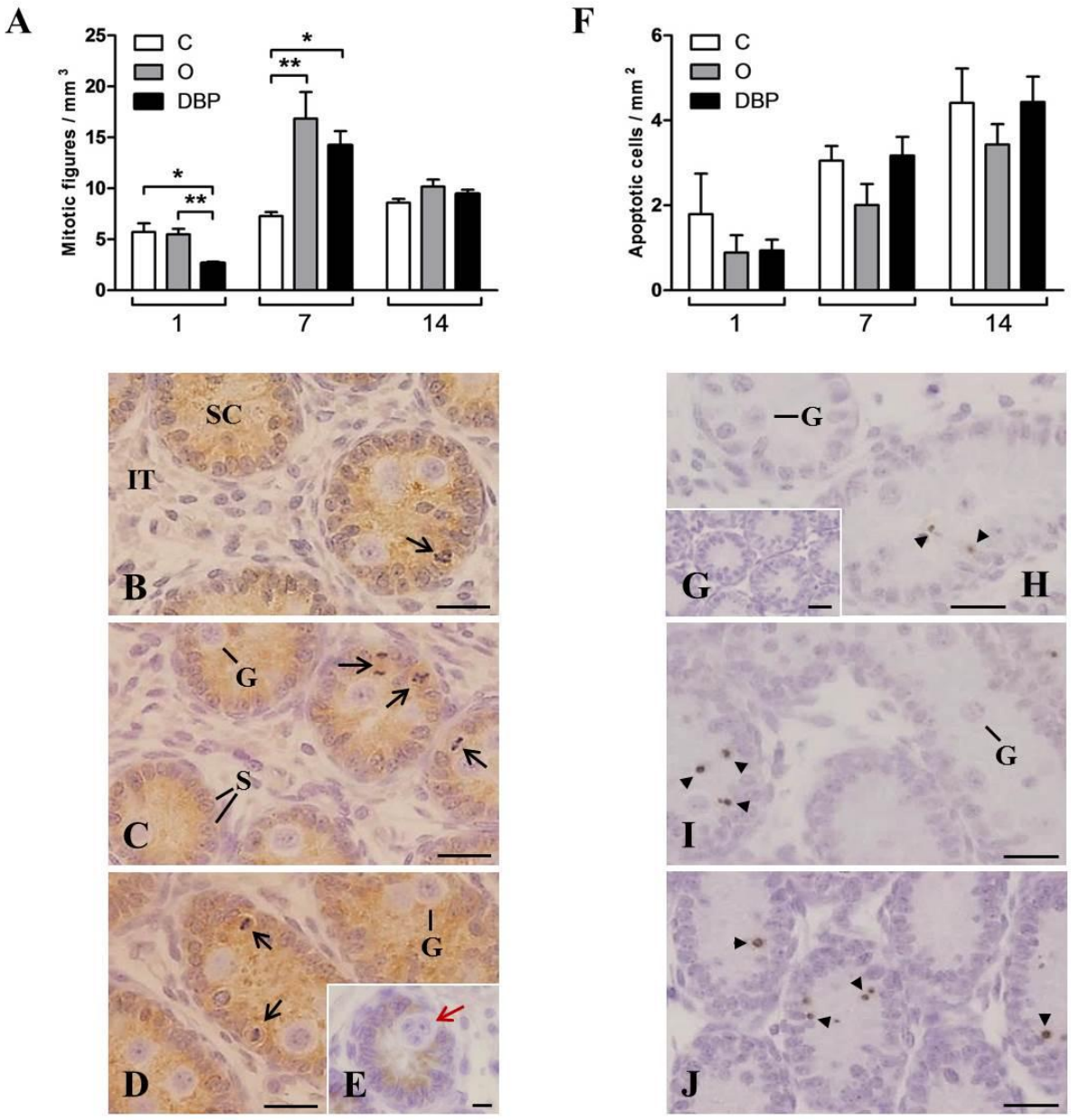

A
C
O
DBP
Mitotic figures / mm 3
25
20
15
10
5
0
1
7
14
*
**
*
**
F
Apoptotic cells / mm 2
6
4
2
0
SC
IT
B
G
S
C
D
E
G
H
I
J

Figure 4

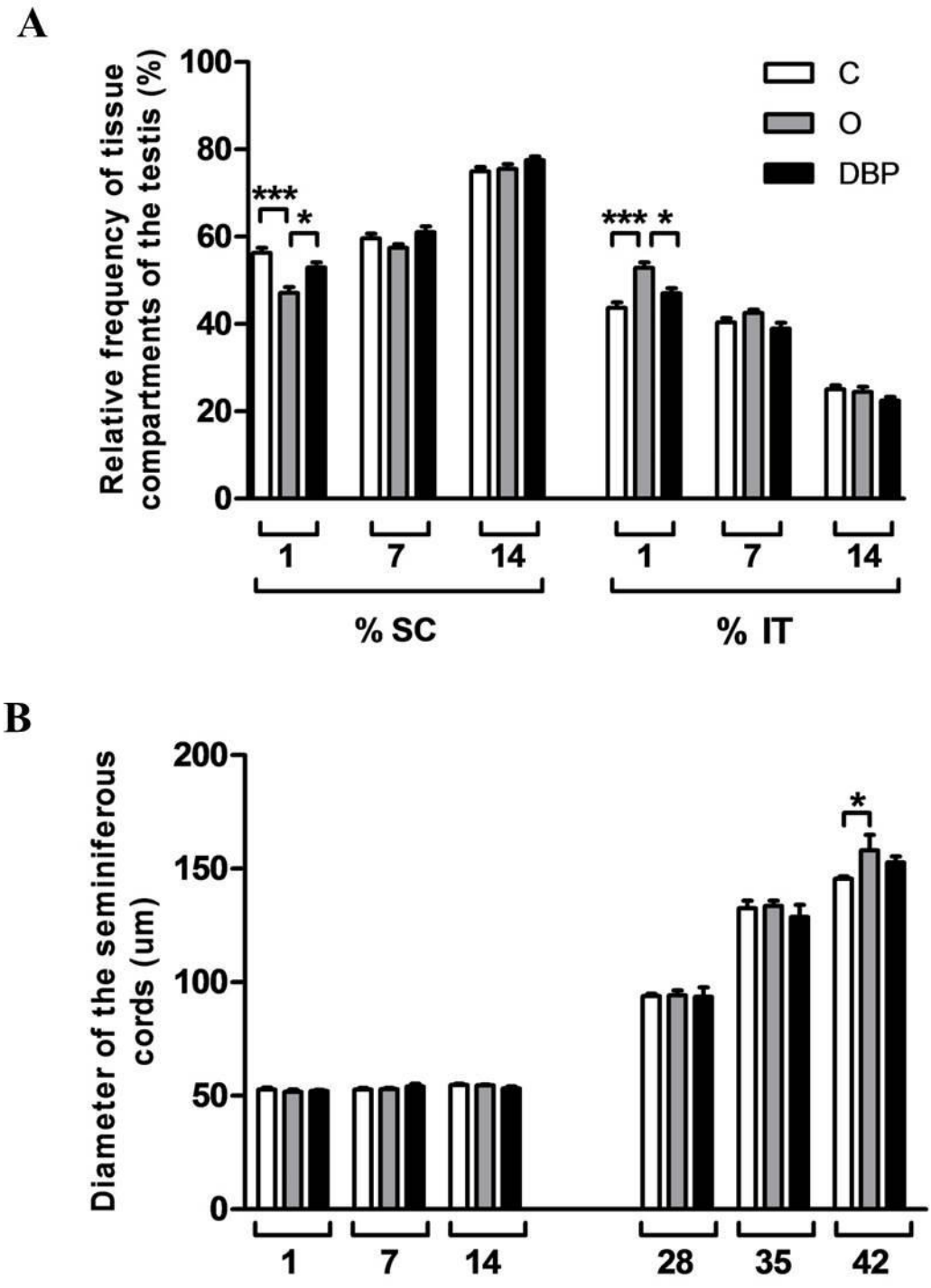

A
Relative frequency of tissue compartments of the testis (%)
100
80
60
40
20
0
C
O
DBP
***
*
***
*
1
7
14
1
7
14
% SC
% IT
B
Diameter of the seminiferous cords (um)
200
150
100
50
0
*
1
7
14
28
35
42

Figure 5

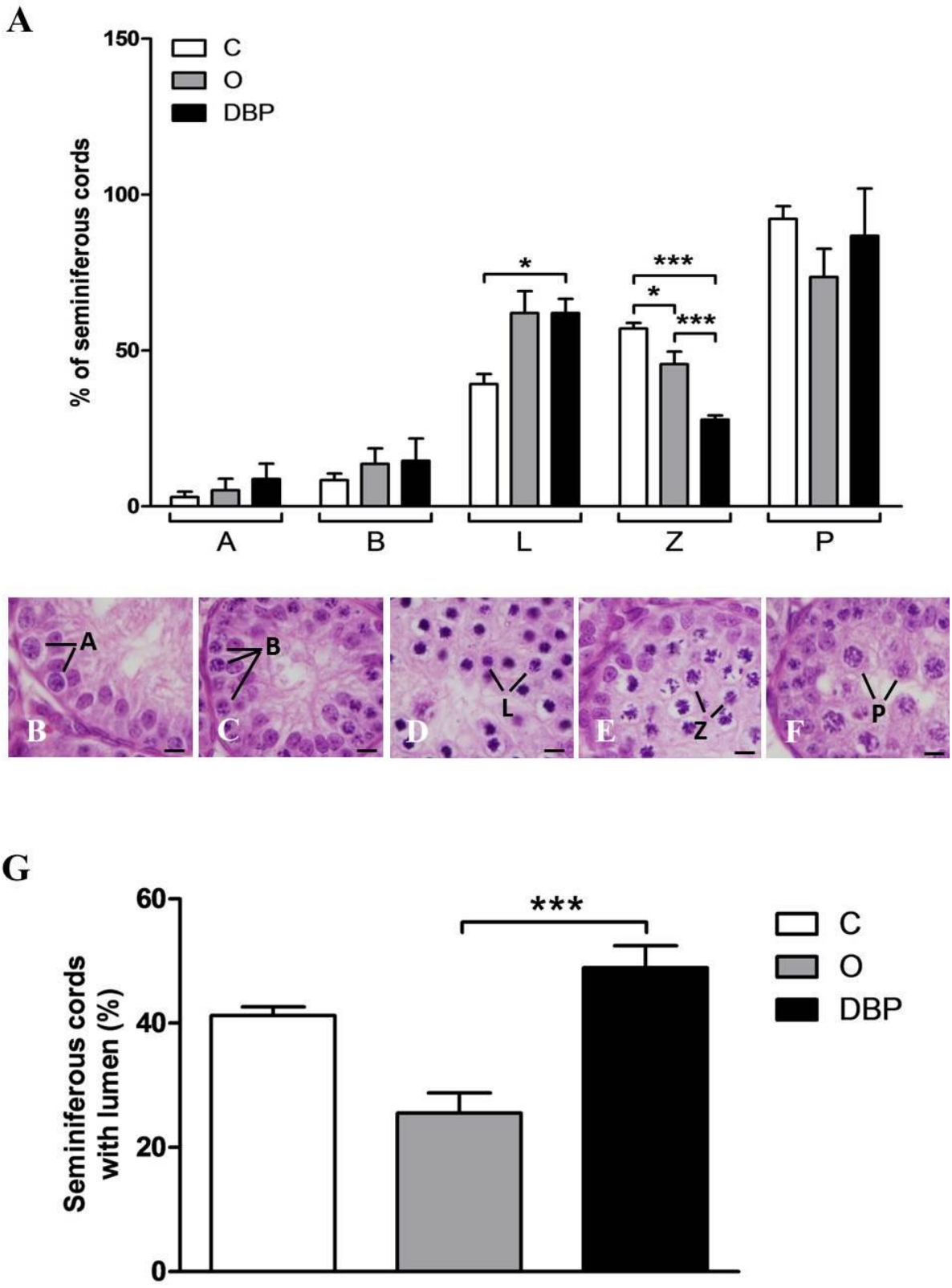
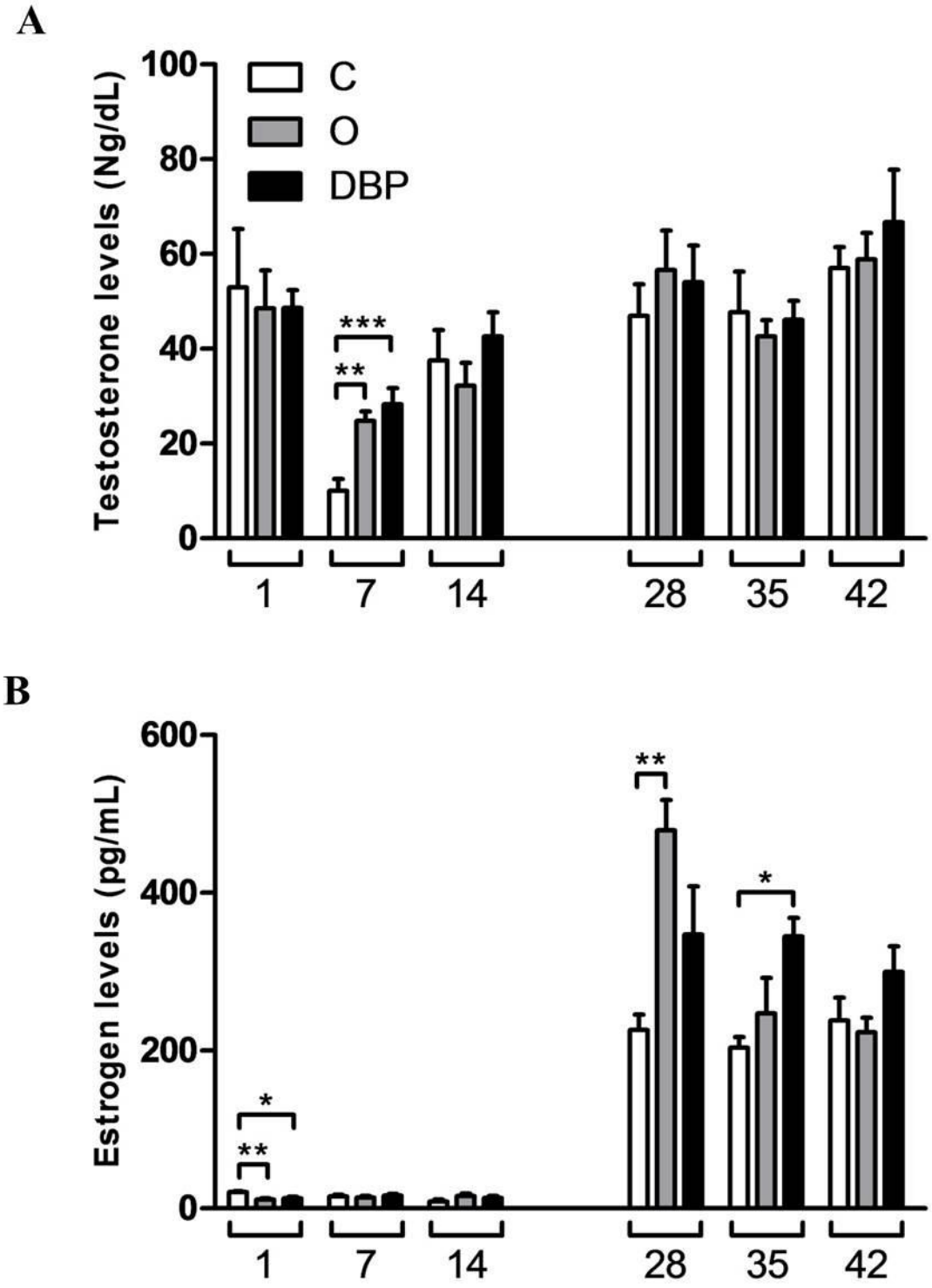

A
150
100
50
0
% of seminiferous cords
C
O
DBP
*
***
*
***
A
B
L
Z
P
A
B
L
Z
P
B
C
D
E
F
G
60
40
20
0
Seminiferous cords with lumen (%)
***
C
O
DBP

Figure 6


A
Testosterone levels (Ng/dL)
100
80
60
40
20
0
C
O
DBP
***
**
1
7
14
28
35
42
B
Estrogen levels (pg/mL)
600
400
200
0
**
*
**
*
1
7
14
28
35
42

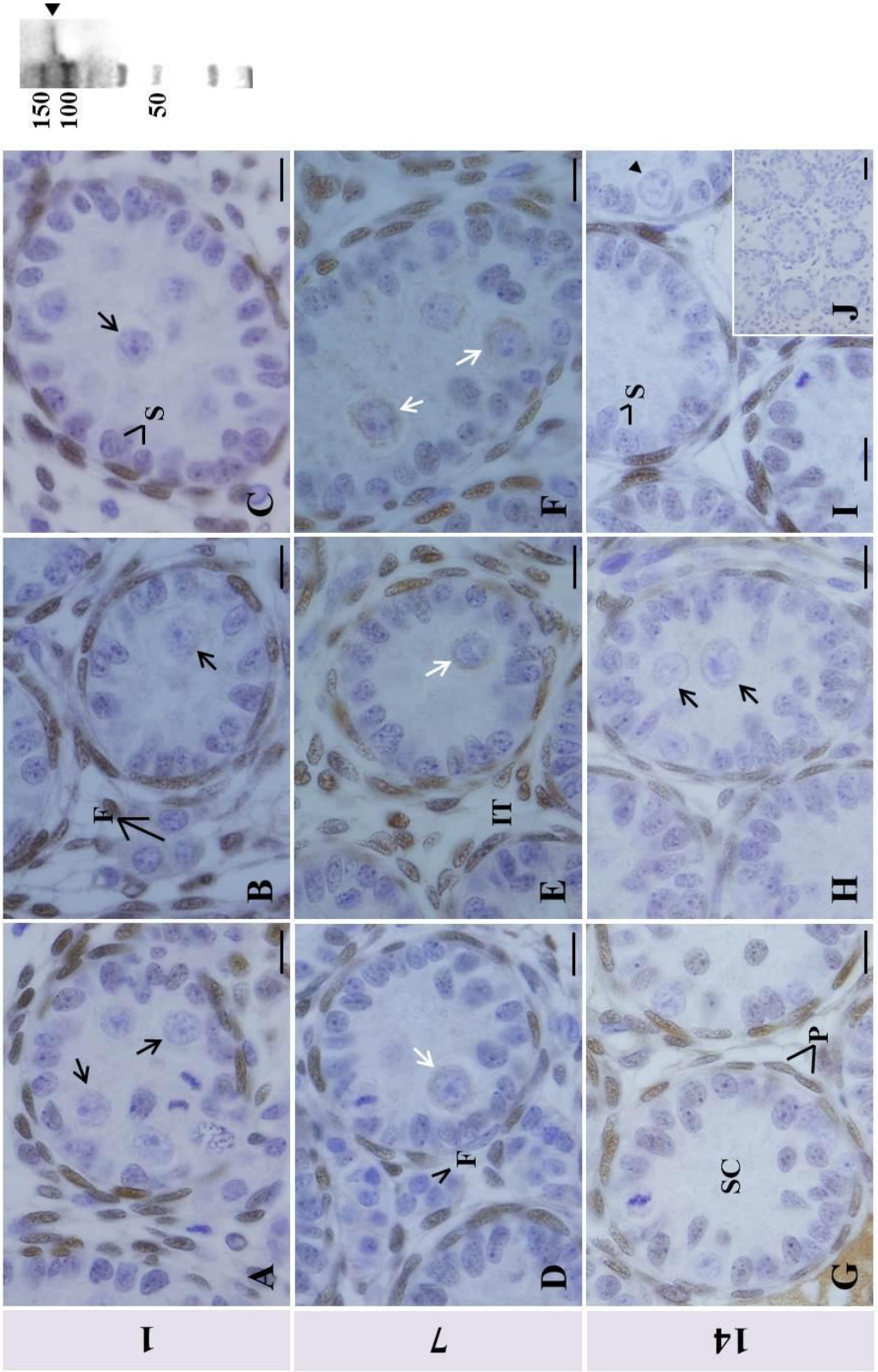
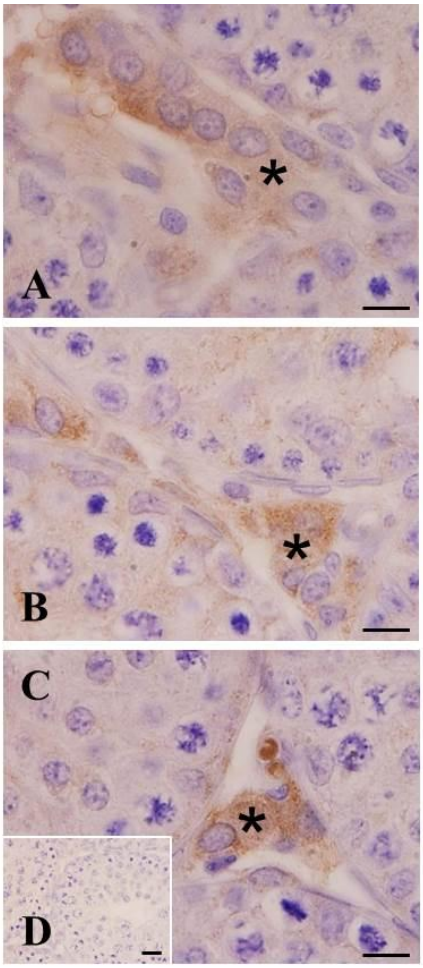
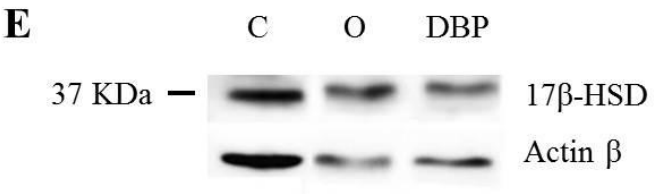
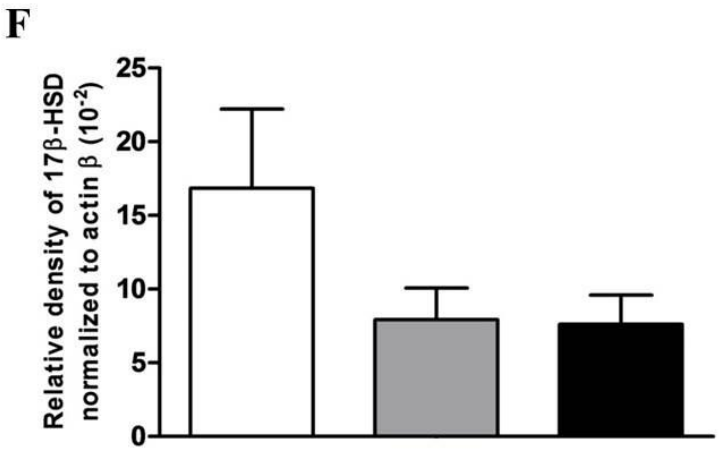


Figure 7

Figure 8


A
B
C
D


E
C
O
DBP
37 KDa
17β-HSD
Actin β


F
Relative density of 17β-HSD normalized to actin β (10-2)
25
20
15
10
5
0

V. MANUSCRITO 2

Docosahexaenoic Acid Alone or in Combination with Mono-(2-ethylhexyl) phthalate Impairs the Mouse Fetal Testis Structure *in vitro* and Increases Testosterone Production

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ABSTRACT

The docosahexaenoic acid (DHA), a long-chain polyunsaturated fatty acid (PUFA), had been recommended as dietary supplementation to pregnant women. In adult male gonad it is beneficial for steroidogenesis, spermatogenesis and sperm motility but the consequences on fetal testis development are unknown. Phthalate esters, such as di-(2-ethylhexyl) phthalate (DEHP) and its active metabolite, mono-(2-ethylhexyl) phthalate (MEHP), exert adverse effects on fetal testis development via alterations in some nuclear receptors (NRs). Gestational exposure to MEHP has been related to deregulation of peroxisome proliferator-activated receptor (PPAR) and also other NRs, such as liver-X receptor (LXR). This study evaluated the action of DHA alone or in combination with MEHP on germ cell populations and steroidogenesis of mouse fetal testis using the organotypic culture system. Mouse testes at 13.5 dpc (days postconception) were cultured for 24 or 72h with medium only (controls), DHA (50µM), MEHP (20µM) or both (50µM of DHA and 20µM of MEHP). After 3d, the total number of gonocytes was not affected by DHA, MEHP or DHA/MEHP. DHA induced a marked extravasation of gonocytes into the interstitial tissue, a pro-apoptotic effect on cell populations in the stroma of the gonad and degenerative alterations in the Sertoli cells, which were worsened when combined with MEHP. This lipid also stimulated testosterone secretion by fetal testis after 72h, regardless incubation with MEHP (control = 52.77 ± 2.35; DHA = 84.88 ± 11.61; MEHP = 53.2 ± 4.95; DHA/MEHP = 101.13 ± 11.71 pg/testis/h). As indicated by qRT- PCR analysis gene expression of *Pparα*, *Pparγ*, *Lxrα*, *Vasa*, *Sox9*, *Fgf9*, *Dmnt3l, 3β-hsd* and StAr were unchanged in samples of whole testis after 24h of incubation in different conditions. The present *in vitro* experiments indicated that DHA exert a proandrogenic action on mouse fetal testis, accompanied by degenerative alterations in the Sertoli cells and extravasation of gonocytes into the the interstitial tissue.

1. INTRODUCTION

The docosahexaenoic acid [22:6 (n-3)] is a long-chain polyunsaturated fatty acid (PUFA) abundantly found in oily fish sources, such as salmon, tuna, and herring (Barbalho et al., 2011; Micha et al., 2014). The profile of PUFAs in the diet and balance between n-3 and n-6 can alter membrane phospholipids and change cell membrane fluidity and physiology (Abbot et al., 2012). Docosahexaenoic acid (DHA) is the major n-3 PUFA in membranes acting as an important regulator of cell signaling, gene expression and inflammatory process (Teitelbaum, Allan Walker, 2001; Akiyama et al., 2013). DHA is also cytotoxic to various cancer cells due to its ability to induce apoptosis (Calviello et al., 1998; Zand et al., 2007).

Maternal DHA dietary supplementation has been recommended because this PUFA contributes for optimal development of central nervous system and it improves developmental outcomes in the offspring (Ozias et al., 2007; Oken et al., 2008; Kar et al., 2016). However, its benefits for both mother and child are still under debate (Makrides et al., 2010; Valentine, 2012; Saccone et al., 2016) and the influence of DHA on testicular development is unknown. This lipid is important for spermatogenesis and sperm motility (Colon et al., 1986; Rejraji et al., 2006; Roqueta-Rivera et al., 2010). In humans, DHA is the main PUFA in sperm and in monkey, 99% of DHA is located in the sperm tail (Connor et al., 1998; Lenzi et al., 2000). A balanced n-3/n-6 PUFA ratio is beneficial to reproduction capacity and is associated with increased testosterone levels, augmented weight and reduced sperm deformity ratio (Yan et al., 2013; Feng et al., 2015).

Phthalates, such as di-(2-ethylhexyl) phthalate (DEHP) and its active metabolite, mono-(2-ethylhexyl) phthalate (MEHP), may exert adverse effects on testis development due to their action on germ cells or steroidogenesis (Sharpe et al., 1995; Mylchreest et al., 1999; Andrade et al., 2006; Howdeshell et al., 2008; Chauvigné et al., 2009; Desdoits-Lethimonier et al., 2012). The *in vitro* exposure of rodent fetal testis to MEHP reduced testosterone synthesis and gonocyte number due to a combination of low proliferation and high apoptosis, without affecting the number of Leydig cells (Chauvigné et al., 2009; Lehraiki et al., 2009).

Both phthalates and DHA may interfere in metabolic pathways involved in cholesterol/lipid synthesis but in antagonistic ways (Hurst, Waxman, 2003; Desvergne et al., 2009; Hatch et al., 2010; Georgiadi, Kersten, 2012; Muczynsky et al., 2012; Kim et al., 2016). Different lines of investigation had shown that phthalate monoesters activated rodent and human peroxisome proliferator-activated receptors (PPARs) (Hurst, Waxman, 2003;

Desvergne et al., 2009; Muczynsky et al., 2012). It was observed, using different cell lines, that MEHP is a more potent PPAR activator than monobenzyl phthalate and mono-sec-butyl phthalate, whereas no significant activation was observed with monomethyl, mono-n-butyl, dimethyl, or diethyl esters (Hurst, Waxman, 2003). Gestational exposure to MEHP have been related to deregulation of PPAR in the male gonad and also other nuclear receptors (NR), such as liver-X receptor (LXR) (Muczynsky et al., 2012). As demonstrated by Muczynsky et al. (2012), MEHP exposure resulted in up-regulation of LXRα and increased expression of seven LXRα downstream genes involved in cholesterol and lipid synthesis, such as the member of Sterol Regulatory Element-Binding Protein (SREBP). In addition, DHA has been reported to play an opposite effect, downregulating the gene transcription of SREBP-1 and enzymes of lipid synthesis (Georgiadi, Kersten, 2012).

Considering the scarcity of knowledge about dietary lipid components on testis development as well as the worldwide recommendation for maternal supplementation, this work examined the effects of DHA individually or in combination with MEHP on fetal testis, using the organotypic culture system, with emphasis on gonocyte development and steroidogenesis.

2. MATERIAL AND METHODS

2.1. Animals

NMRI (Naval Maritime Research Institute) mice or NMRI Oct4-GFP mutant mice for use in this experiment were housed under controlled photoperiod (lights on 08:00-20:00) with *ad libitum* access to tap water and diet. Males were caged with females overnight and the day following overnight mating was counted as 0.5 dpc (days post coitum). Pregnant mice were killed by cervical dislocation on 13.5 dpc and the fetuses were quickly removed from the uterus and dissected under a binocular microscope. Their sexes were determined on the basis of the gonad morphology and the testes collected from male fetuses. All animal studies were performed in accordance of NIH Guide for Care and Use of Laboratory Animals. The animal facility is licensed by the French Ministry of Agriculture (agreement N°B92-032-02).

2.2. Organ culture and treatments

Organ cultures were conducted as previously described (Habert et al., 1991; Livera et al., 2006; Lehraiki et al, 2009). The whole testes from 13.5 dpc mice, separated from the mesonephros, were placed on 12-mm-diameter Millicell CM filters (pore size 0.45μm) in

tissue culture dishes and incubated at 37°C, in humidified atmosphere containing 95% air / 5% CO_2 and were floated on 320µL DMEM/F-12 culture medium (Gibco, Scotland, UK, 11039-021) in 24-well tissue culture plates, supplemented with gentamicin 80 µg/ml (Gibco, New York, USA, 15750-037). Testes were cultured for 24 or 72 hours with 0.1% DMSO (Sigma Aldrich, Missouri, USA, D2650) only (control), or with 20µM of MEHP (TCI Europe, Antwerp, Belgium), or with 50µM of DHA (Sigma Aldrich, Missouri, USA, D2534) or with both (20µM of MEHP plus 50µM of DHA). The medium was changed every 24h. Testes of male fetus from 6 pregnant mice cultured for 72h (n = 7 to 9 per group) were fixed for 24h at room temperature in 10% formalin, embedded in paraffin and cut into 5µm sections. Testes of male fetus born from 16 female mice cultured for 24h were placed in RTL buffer with Qiagen kits for RNA analysis, or immersed in collagenase/DNAse solution for cell sorting analysis. For the real-time qPCR of the whole testis they were used pools of 4 to 5 testes per group and for sorted cells, pools of 3 to 6 testes per group.

2.3. Immunocytochemistry

Serial sections were subjected to double immunostaining for VASA, using a mouse monoclonal anti-DDX4/MVH (Abcam, ab27591; dilution 1:200, for 1h at 37°C) and for cleaved caspase-3, using a rabbit polyclonal anti-cleaved caspase-3 (Cell signaling, 96615; dilution 1:200, for 12h at 4°C). Cleaved caspase-3 immunolocalization was identified using 3,3′-diaminobenzidine (DAB - Vector Laboratories) and then VASA localization was detected using VIP substrate (Vector Laboratories). All the gonocytes in every fifth section were counted using Histolab analysis software (Microvision Instruments, Evry, France). The total number of gonocytes was multiplied by 5, as appropriate, to obtain the crude count (CC) of gonocytes per testis. The Abercrombie formula was used to correct for double-counting attributable to the appearance of a single cell in two successive sections: $TC = CC \times S/(S+D)$ where TC is the true count, S is the section thickness (5µm) and D is the mean diameter of the gonocyte nuclei (Abercrombie, 1946). The mean nuclear diameter measured on the section (DM) was divided by $\pi/4$ to correct for the over-representation of smaller profiles in sections through spherical particles. The value DM was measured on each testis studied, by means of at least 40 random determinations. The apoptotic index was estimated after immunoreaction to cleaved caspase-3, by counting the same sections used in the total number of gonocytes determination. The number of cleaved caspase-3 positive cells inside or outside the seminiferous cords was calculated, per section, by the formula: (caspase-3^+ inside or caspase-

3^+ outside the seminiferous cords/total number of caspase3^+ cell) x 100. The index of gonocytes located outside the testicular cords and the index of multinucleated gonocytes were obtained in the same manner as described for apoptotic cells. Quantitative analyzes were performed for 3 (Control, DHA, DHA/MEHP) or 4 (MEHP) testes per group.

Testicular volume was determined by the Cavalieri method (Gundersen et al., 1988). One of the 5 histological sections had its area determined using Histolab analysis software (Microvision Instruments, Evry, France), and testicular volume was calculated by multiplying the sum of the areas by 25, a value that is related to the distance between two successive sections (5 x 5µm).

The Sertoli cells were identified after immunocytochemical staining for GATA4 (goat polyclonal anti-GATA4; Santa Cruz, sc-1237; dilution 1:200, for 12h at 4°C). GATA4 localization was then detected with DAB (Vector Laboratories) as the substrate.

The proliferating cells were identified using the method of BrdU (5-bromo-2#-deoxyuridine) incorporation. BrdU (1%) was added to the culture medium 3h before the end of culture. The sections were subjected to double immunostaining for BrdU, using antibody anti-Bromodeoxyuridine (Sigma Aldrich, BMC9318 clone, 11170376001; dilution 1:100, for 1h at 37°C), and for anti-Müllerian hormone (AMH) (goat polyclonal anti-MIS; Santa Cruz, sc-6859; dilution 1:200, for 12h at 4°C). BrdU incorporation was localizated using DAB (Vector Laboratories) and then AMH was detected using VIP substrate (Vector Laboratories), for identification of Sertoli cells. These procedures were performed according to the manufacturer's recommendations and detailed by Lambrot et al. (2006). The BrdU incorporation index (percentage of cells showing a clear positive immunoreaction to BrdU) was obtained by a blind counting of 1000 cells inside the seminiferous cords and 1000 cells in the interstitial tissue on the sections (3 per group).

All antibodies used in immunocytochemical reactions were diluted in 2.5% Normal Horse Serum Blocking Solution (Vector Laboratories).

2.4. Cell sorting analyses

Testis cultured for 24h were used in these assays. Briefly, for cell dissociation, cultured testes were placed in PBS supplemented with 100 mg/mL of collagenase I (NB 4, Clostridium histolyticum) and 0.2 mg/mL DNase I (Sigma Aldrich, D5025) for 15min at 37°C, with repeated pipetting. Dissociated cells from transgenic animals (NMRI Oct4-GFP mutant mice) were subjected only to immunostaining with CD49 (rat anti-human CD49; BD

Pharminger, 555736 – 1:100), a specific antigen of Sertoli cells, and in the case of normal animals (NMRI mice) immunostaining was also performed to the CD9 (biotin anti-mouse CD9; BD Pharminger KMC8, 558749 – 1:100), specific antigen of gonocytes, followed by incubation with streptavidin (Vector Laboratories, AS 5488, DyLight 488 – 1:200). Cells were then centrifuged (10 min at 1000G) to remove the dissociation solution and suspended in blocking solution (5% BSA in PBS). Sorting was performed using BD FACSAria Fusion™ flow cytometer (BD Biosciences) with laser (laser = 488 nm for GFP and CD9, and 407 nm for CD49-PE), in wavelength and filter of 530/30 for CD9 and GFP, and 427/10 for CD49-PE. Thus, three cell populations were separated - Sertoli cells, germ cells (gonocytes) and the remaining cells (fibroblasts, pericytes, mesenchimal cells and Leydig cells). These cell populations were recovered in RLT buffer (Qiagen, Courtaboeuf, France) for RNA extraction and analysis of gene expression.

2.5. Quantitative Real-time Polymerase Chain Reaction (q-RT PCR)

The sorted cell populations or pools containing 5 or 6 whole testis per group were transferred to RLT buffer and lysed. Total RNA was extracted using the RNeasy minikit (QIAGEN, Valencia, CA), quantified using NanoDrop ND-100 and stored at -80°C. Reverse transcription was achieved using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The qPCR reactions were carried out and the fluorescence detected using StepOnePlus system (Life Technologies). The comparative ΔΔcycle threshold method was used to determine the relative quantities of mRNA, using *actin β* mRNA as the endogenous reporter. The gene expression of control groups were defined as 100% and for the other groups the expression was presented in relation to control. Each RNA sample was analyzed in duplicate. All primers were used at a final concentration of 400 nM. Sequences of oligonucleotides used are given in Table 1.

Table 1: Primers used in Real-Time RT-PCR

Gene	Code primer	Species	Orientation	Sequence 5'-3'
Actinβ	mActineB	mouse	Forward	GCCCTGAGGCTCTTTTCCAG
Actinβ	mActineB	mouse	Reverse	TGCCACAGGATTCCATACCC
Fgf9	mFGF9_596	mouse	Forward	ATCTTCCCCAACGGTACTATCCA
Fgf9	mFGF9_696	mouse	Reverse	CCGCGAATGCTGACCAG
Sox9	Sox9	mouse	Forward	GGCAAGCTCTGGAGGCTG
Sox9	Sox9	mouse	Reverse	CCTCCACGAAGGGTCTCTTCT
Srebp1c	MMSREBP1-C	mouse	Forward	GTGAGCCTGACAAGCAATCA
Srebp1c	MMSREBP1-C	mouse	Reverse	GTGCCTACAGAGCAAGAGG
PparϒΥ	mPPARg_741	mouse	Forward	TGTCTCACAATGCCATCAGGTT
PparϒΥ	mPPARg_791	mouse	Reverse	TCTCCTTCTCGGCCTGTGG
Pparα	mPPARa_542	mouse	Forward	ACCACTACGGAGTTCACGCAT
Pparα	mPPARa_592	mouse	Reverse	TTCGCCGAAAGAAGCCCT
Lxrα	MMLXR	mouse	Forward	GGCTGCAGGTGGAGTTCATC
Lxrα	MMLXR	mouse	Reverse	AATGAGCAGAGCAAACTCAGCAT
Vasa	mVasa	mouse	Forward	GAAGAAATCCAGAGGTTGGC
Vasa	mVasa	mouse	Reverse	GAAGGATCGTCTGTCTGAACA
Dmnt3l	mDmnt3L	mouse	Forward	GGCACATAACCCTCCCTCAAA
Dmnt3l	mDmnt3L	mouse	Reverse	CGAAGTCAAAGTGAACCGACG
Cyp11A1	mCyp11A1_620F	mouse	Forward	TTCCGCTTTTCCTTTGAGTCC
Cyp11A1	mCyp11A1_695R	mouse	Reverse	GATCCACGATCTCCTCCAGC
3bHSD	3bHSD_m_S	mouse	Forward	TGGTGACAGGAGCAGGA
3bHSD	3bHSD_m_AS	mouse	Reverse	AGGAAGCTCACAGTTTCCA
StAR	m_Star_F	mouse	Forward	CCGGAGCAGAGTGGTGTCA
StAR	m_Star_R	mouse	Reverse	CAGTGGATGAAGCACCATGC

2.6. Measurement of testosterone production

Testosterone secretion by fetal testis was evaluated after 24 and 72h of culture by quantification of this hormone in the culture medium. Medium samples were analyzed in duplicate using direct radioimmunoassay without extraction as previously described (Habert, Picon, 1984).

2.7. Statistical analysis

Statistical analyses were performed with GraphPad 5.0 (© 1992-2007 GraphPad Software, Inc.) by T-test or Mann-Whitney test. mRNA expression analysis were performed using Wilcoxon signed-rank test for comparison with the control group and the other groups (DHA, MEHP and DHA/MEHP). Data are presented as individual values; bars indicate mean and SEM. $p \leq 0.05$ was considered significantly different.

3. RESULTS

3.1. DHA induces degenerative changes in Sertoli cells and disorganization of the testicular cords, which are aggravated by MEHP

The incubation with 50µM of DHA apparently did not alter the histological organization of the mouse fetal testes, after 72h, at 40x magnification (Fig. 1A). However, at 100x objective, testicular cords with abnormal features were observed in 50% of explants. These cords were disorganized, showing an apparent rupture of the basement membrane (Fig. 1E, F) and a collective degeneration of Sertoli cells as evidenced by pyknotic nuclei (Fig. 1B, D). Morphological signs of cord degeneration were also observed after exposure to DHA plus MEHP (Fig. 2D). In this case, the analysis of serial sections of the testis indicated a normal organization in the organ periphery but a severe necrosis occurred in the central area. This necrosis was so marked for 60% of cultured testis, which assumed a cup shape (Fig. 2A). High amounts of necrotic, apoptotic and proliferating cells were observed in the boundaries of degenerating central areas (Figs. 2A", A'", B, E, F). Immunostaining for GATA4 indicated the presence of this transcription factor in the nuclei of the Sertoli cells and in low amounts in those of the stromal cells of all groups considered (data not shown for control and MEHP groups). This immunostaining confirmed that DHA causes a disorganization of Sertoli cells and the dispersion of them into the interstitial tissue (Fig. 1E, F). The testicular cords in the periphery of the testes incubated with DHA/MEHP maintained the typical organization of this cell type as well as nuclear labeling for GATA4 (Fig. 2B) but the combined exposure led to

loss of GATA4 in the nuclei of some stromal cell clusters (Fig. 2C). Despite the morphological changes seen for DHA and DHA/MEHP groups, the volume of the gonads did not change among groups (control = 0.028 ± 0.004 mm; DHA = 0.026 ± 0.001 mm; MEHP = 0.026 ± 0.004 mm; DHA/MEHP = 0.028 ± 0.004 mm).

3.2. DHA increases the percentage of ectopic gonocytes without affecting the total number of these cells in the fetal testis

Quantitative analysis revealed no statistical difference in the total number of gonocytes in the fetal testis among groups (Fig. 3A). DHA increased significantly the percentage of gonocytes located outside the seminiferous cords in relation to the control and MEHP groups (Fig. 1C, D; 3B). It was also observed for the incubation with MEHP an increase of multinucleated gonocytes located in the interstitial tissue in relation to the control (Fig. 3C). Testis exposed to DHA/MEHP showed a reduction of 25% in the percentage of MNG inside the cords accompanied by an increase, in the same proportion, of MNG in the interstitial tissue also in relation to the control groups (Fig. 3C).

The percentage of sorted cells did not vary among groups for any of the three populations separated: germ cells (gonocytes), Sertoli cells and the remaining cells of the fetal testis (Fig. 4). Considering the germ cell counts and the percentage of sorted germ cells the pattern observed for the groups C, DHA, MEHP and DHA/MEHP were quite similar (Fig 3A; Fig. 4).

3.3. DHA increases apoptosis in the stromal compartment and the proliferation index of Sertoli cells both alone or combined with MEHP

Analysis of the cleaved caspase-3 labeled cells revealed that DHA significantly decreased the apoptosis inside the testicular cords and increased it in the interstitial tissue in relation to the control group (Fig. 5A). This was also observed for the incubation with DHA/MEHP an increase of 57.5% in the percentage of cleaved caspase-3 positive cells in the interstitial region of the gonads and a decrease of 48.5% of these cells in the testicular cords compared to the control (Fig. 5A).

The measurement of BrdU Incorporation Index, obtained after double immunostaining for AMH and BrdU, did not vary in the Sertoli cells located in the interstitial tissue for any group (Fig. 5B). On the other hand, exposure to DHA/MEHP increased the proliferation index of the remaining cell populations outside the testicular cords in 15% in comparison with the

control group. The combined incubation also increased the proliferation of Sertoli cells inside the cords in 11.5% when compared to the control. Still in relation to the testicular cords, Sertoli cell proliferation was 8% higher in DHA exposed group compared to the control (Fig. 5B). No labeling was observed for the germ cells (data not shown).

3.4. Effects of incubation with DHA, MEHP and DHA/MEHP on gene expression of 11 different genes involved in the development and differentiation of the fetal testis

The expression of the nuclear receptors *Pparα*, *Pparγ* and *Lxrα*, of *Vasa*, *Sox9*, *Fgf9* and *Dmnt3l* and of the steroidogenic enzymes *3β-hsd*, *StAR and Cyp11a1* was analyzed in samples of whole testis after 24h of culture (Fig. 7). Although it was not statistically significant, the qRT-PCR indicated that DHA decreased *Fgf9* and *Pparα* expression and increased *Cyp11a1* expression in relation to the control. Except for the decrease in *Pparα* these alterations do not appear to occur when DHA is combined with MEHP (Fig 7). The analysis of gene expression after cell sorting indicated that the remaining cell populations (RC) of testis, containing the different stromal cell types, including Leydig cells, was more affected than gonocytes and Sertoli cells (Fig. 8). It was observed an increase in the expression of *Fgf9*, *Pparα* and *Srebp1* in RC, for DHA, MEHP and DHA/MEHP groups, in relation to germ cell and Sertoli cell populations (Fig. 8).

3.5. DHA individually or combined with MEHP increased testosterone secretion after 72h of culture

The incubation of fetal testis with DHA individually or in combination with MEHP did not alter testosterone secretion after 24h in relation to the control (Fig 6A) but increased it after 72h (Fig. 6B). MEHP did not affect testosterone secretion in dose and time periods evaluated when compared to the control group (Fig. 6).

4. DISCUSSION

The possible modulation of the male gonad during gestation by specific dietary lipids is unknown. Therefore, in this study, we used a closed system of organotypic culture to investigate the direct effects of DHA on mouse fetal testis development, its action on different testicular targets as well as its interference on alterations induced by MEHP. Our data indicated that DHA caused a subtle disorganization of testicular cords with signs of degeneration of Sertoli cells and a marked extravasation of gonocytes into the interstitial

tissue. Thus, although the total number of gonocytes had not be affect by incubation with DHA, four folds more ectopic gonocytes were observed compared to the control group, indicating impairment in the germ cells development by this PUFA. Our results also demonstrated, for the first time, a stimulatory action of DHA on testosterone production by mouse fetal testis after 72h of culture.

The consequences of phthalate expose on steroidogenesis of fetal testis have previously been examined using this organotypic culture and the results indicate a species- and a dose-specific effect. Thus, 10^{-5} M of MEHP reduced testosterone production of 14.5 dpc rat testis after 72h, whereas the same dose of DEHP increased it (Chauvigné et al., 2009). The exposure of human fetal testes to MEHP resulted in no change in testosterone production, even in higher doses (10^{-4}M; Lambrot et al., 2009). Unlike demonstrated in our study, Lehraiki et al. (2009) observed an increase in testosterone secretion by mouse fetal testis cultured for 72h, with 200µM MEHP, probably due to the higher dose of phthalate than that used by us. Then, the present results reinforce the dose specific effect of MEHP on steroidogenesis, since low MEHP dose induced no alteration in testosterone secretion after 3d of culture. On the other hand, DHA induces a marked increase in testosterone secretion by fetal testis, by mechanisms that are not affected by MEHP. Diet-supplemented with DHA increased the concentrations of testosterone in plasma of male buffalo and male rams (Esmaeili et al., 2014; Tran et al., 2016). The balanced n-3/n-6 PUFA ratio in diet also increased testosterone levels in rats at 90 days by increasing GnRH, LH and FSH in serum, and in young breeder rooster for enhancing mRNA levels of GnRHR, FSHR, LHR and StAR (Yan et al., 2013; Feng et al., 2015). Recently, Mohammad et al. (2015) demonstrated that DHA enhances testicular dysfunction induced by chloride ($AlCl_3$) by mechanisms related to their potent antioxidant potential and stimulatory effects on steroidogenic enzymes transcription (*3β-hsd*, *17β-hsd* and *StAR*). Taken together, previous reports with different adult mammals suggest that DHA in diet may favor testis steroidogenesis by different mechanism. However, to the best of our knowledge, this is the first evidence of an androgenic action of this PUFA to the fetal testis. DHA did not alter the expression of *StAR, 3β-hsd*, and *Cyp11a1* after 24h of incubation but we cannot rule out the interference of DHA in the regulation of these enzymes, considering that this may occur in longer incubation times.

The formation of multinucleated gonocytes (MNG), an usual consequence of exposure to endocrine disruptors, was reported to the mouse (Gaido et al., 2007) and rat (Ferrara et al., 2006) fetal testis at 19 dpc after maternal exposure to DBP, and to the mouse testis at 15.5 and

18.5 dpc after *in vitro* exposure to higher doses of MEHP (200µM) than that used in the present study (Lehraiki et al., 2009). Here it was demonstrated that induction of multinucleated gonocytes also occurred in mouse fetal testis at 13.5 dpc after 72h of culture with lower doses of MEHP (20µM). A recent study in which Sprague Dawley rats were exposed to 500 mg/kg of di-n-butyl phthalate at 17 or 18 gestational days, following the estimation of proliferation and apoptosis, revealed that the formation of multinucleated gonocytes is a degenerative and not a proliferative process (Spade et al., 2015). An increased percentage of MNG was observed in MEHP group compared to the control group in the interstitial tissue of the testis. The combined incubation also disturbed the gonocytes development with regard to the appearance of MNG. Fetal exposure to DHA/MEHP resulted in a 25% downward trend of this cell type inside the cords and in the same percentage of increase in the interstitial tissue in relation to the control groups. Thus, multinucleated gonocytes were a consequence of incubation with phthalate and not with DHA

Although recent studies correlate the consumption of DHA with improvement of testicular function and reproductive ability (Esmaeili et al., 2014; Tran et al., 2016), Leat et al. (1983) observed a reduction in testicular size and degeneration of the seminiferous tubules, with progressive loss of germ cells in rats at 4 weeks of age fed a diet supplemented with 18:3ω3. The degenerative changes in the tubules was related to lower docosapentaenoic acid (22:5ω6) in testicular lipids. Since 22:5ω6 is an important lipid found in spermatids and spermatozoa, this could imply a decrease in the number of these germ cells and morphological alterations in the testis of animals fed a diet supplemented with this PUFA (Leat et al., 1983).

DHA also exerted an adverse effect on seminiferous cord resulting in degeneration of Sertoli cells and exit of gonocytes to the interstitial tissue besides a pro-apoptotic effect to cell populations in the stroma of the fetal testis after 72h of incubation with this lipid. The increased apoptosis in cells located in the stromal compartment may be triggered by perturbation in their microenvironment. In addition, a proapoptotic action of DHA had been shown to hepatocarcinoma 3924A cells transplanted in ACI/T rats (Calviello et al., 1998) and hematopoietic cells (Zand et al., 2007). According to Zand et al. (2007), DHA induces apoptosis of hematopoietic cell lines via *Pparγ* activation and up-regulation of *p53*. Here, DHA did not alter *Pparγ* expression but increased the expression of *Pparα*. The action of DHA on cell behavior is complex and may occur by direct mechanisms via activation of NRs PPAR and RXR or G protein-coupled receptors (GPR) 40–43 and GPR120 or via their derivatives, such as eicosanoids (Georgiardi and Kerstern, 2012). The analysis of expression

of several genes performed here, using a pool of testis from three different experiments, did not evidenced significant alterations due to variability of data. Then, we could not conclude a specific effect of this PUFA on the listed genes.

Exposure to DHA seems also interfered with Sertoli cells differentiation since many testicular cords did not express GATA4 and clusters of GATA4-negative Sertoli cells were seen in interstitial tissue of testis cultured with DHA/MEHP. GATA4 is a transcription factor involved in gonadal differentiation, sex determination and normal Sry expression (Tevosian et al., 2002). It is also involved in the establishment and maintenance of the spermatogonial stem cells (SSC). Depletion of GATA4 alters chemokine signaling factors which guides SSCs or gonocytes to the stem cell niche (Chen et al., 2015) and leads to a delay in the development of Sertoli cells (Tevosian et al., 2002). Thus, this loss of GATA4 can be related to failure differentiation of Sertoli cells, consequent malformation of the testicular cords and extravasation of gonocyte into the interstitial tissue.

Although the expression of *Pparγ* was not altered by exposure to DHA, it is also known that this PUFA can antagonize the activity of *Lxrα* and down regulating the gene expression of *Srebp-1* (Georgiadi, Kersten, 2012). In the present study, *Srebp-1* expression was 41% higher in the DHA group compared to DHA/MEHP group. This data shows that DHA probably prevented the stimulation of MEHP on mRNA expression of *Srebp-1* (Muczynski et al., 2012).

In addition to the pro-apoptotic effect, the combined incubation also increased the proliferation index of remaining cells (cells others than gonocytes and Sertoli cells) in the stroma of the fetal testis. But unlike that observed for the percentage of apoptotic cells, incubation with DHA/MEHP increased the proliferation in the testicular cords. However, considering that no BrdU-positive gonocytes were found in the testis at 13.5 dpc, the decrease of apoptosis inside the testicular cords after incubation with DHA or DHA/MEHP did not result in change in the total number of gonocytes.

The exposure to 20µM of MEHP did not affect the total number of germ cells, unlike that observed by Lehraiki et al. (2009). These authors demonstrated a decrease in the total number of gonocytes of 13.5 dpc mouse fetal testis after 72h of culture in the presence of 200µM of MEHP, beyond a marked increase in testosterone secretion. Using testis from mouse deficient for estrogen (ERαKO and ERβKO) and androgen (Tfm) they concluded that MEHP effects were independent from androgen and estrogen signaling.

Surprisingly, exposure to DHA/MEHP resulted in severe damage to the testicular structure after 3 days of culture. Although the periphery of the testis showed a normal organization, a large central area of necrosis was seen in most of the cultured gonads, making them to assume a cup shape, never observed before with this organotypic culture system. This degenerating lesion was related to induction of apoptosis and necrosis. It should be noted that these areas were not used in the estimation of proliferative and apoptotic cells. In a recent study conducted in our laboratory it was found that DHA (100µM for 48 h) decreased the mitochondrial energy reserve capacity, inhibited the AKT and mTOR oxidative stress pathways and increased the cell death of human luminal prostate normal cell line PNT1A (Tamarindo et al., unpublished data). We don't know as DHA is metabolized in the testis but on the basis of its action on mitochondrial oxidative stress and cell death it is possible that the deleterious effects of incubation with DHA reached the threshold for cytotoxic effects when in association with MEHP, leading to tissue degeneration.

In conclusion, the present results showed that DHA impairs the mouse fetal testis development, beyond stimulating testosterone secretion. This PUFA induced degenerative alterations in Sertoli cells, which were worsened when combined with MEHP, and extravasation of gonocytes into the interstitial tissue. The indications provided by these *in vitro* experiments strengths about the relevance of investigating the *in vivo* effects of this lipid and the consequences of maternal DHA intake for the fetal testis development and posterior reproductive capacity.

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LEGEND OF FIGURES

Figure 1. Histological sections of testes of mouse at 13.5 dpc subjected to double immunostaining for VASA and cleaved caspase-3 (A-D) and for GATA4 (E, F) after 72h of culture in the presence of 50µM DHA. A: Panoramic image showing the normal morphology of the testis. B: Degenerating testicular cord with pyknotic nuclei on the left C: Gonocytes localized outside the testicular cords. D: Area with collective degeneration of Sertoli cells. E, F: Disorganized testicular cords. Legend: Arrows – gonocytes located outside the seminiferous cords. Bars = 10µm (B-F) and 200µm (A).

Figure 2. Histological sections of testes of mouse at 13.5 dpc subjected to double immunostaining for VASA and cleaved caspase-3 (Fig. 2A, E) and for BrDU and anti-Müllerian hormone (Fig. 2D, F), and immunostaining for GATA4 (Fig. 2B, C) after 72h culture in the presence of 50µM DHA and 20µM of MEHP. A: Serial sections showing a normal morphology of testis (A') followed by a loss of testicular cords in the center (A'') and finally the presence of a hole inside the gonad (A'''-A''''). B: Inner side GATA4-negative (arrowheads). C: Cluster of GATA4-negative cells (asterisk). D: Disorganized testicular cord on the right. E: Apoptotic cells in the center of the gonad (black arrows). F: BrDU-positive cells in the center of the testis (red arrows). Bars = 200µm (A), 50µm (B), 20µm (C-D) and 10µm (E-F).

Figure 3. (A) Total number of gonocytes and (B) Percentage of ectopic gonocytes and (C) Multinucleated gonocytes located in the testicular cords (TC) or in the interstitial tissue (IT) of control testes or testes incubated with DHA (50µM), MEHP (20µM) or both (50µM DHA and 20µM MEHP) after 72h of culture. Histograms represent the mean ± SEM of 3 (Control, DHA and DHA/MEHP) or 4 (MEHP) different testis from different fetuses for each condition.

Figure 4. (A) Cell sorting profiles and (B) Percentage of germ cells (GC), Sertoli cells (SC) and remaining cells (RC) populations after 24h of culture with DHA (50µM), MEHP (20µM), DHA/MEHP (50µM and 20µM, respectively) or medium only (control) in the fetal testis (13.5 dpc). Legend: a - Oct4-positive cells (germ cells); b - CD49-positive cells (Sertoli cells);

c - unlabeled cells (remaining testicular cell populations). Histograms represent the mean ± SEM of 5 different experiments of cell sorting.

Figure 5. (A) Percentage of cleaved caspase-3 and (B) Proliferation index in mouse fetal testis after 72h of culture with DHA (50µM), MEHP (20µM), DHA/MEHP (50µM and 20µM, respectively) or medium only (control). Legend: IT – interstitial tissue; RC – remaining cells (cells other than gonocytes and Sertoli cells); SC – Sertoli cells; TC – testicular cords. Histograms represent the mean ± SEM of 3 different testes from different fetuses for each condition. For the determination of apoptosis index they were used 4 testes in MEHP group.

Figure 6. (A) Testosterone secretion in fetal testis after 2 h (4 to 6 samples per group) or (B) 72h (6 to 9 samples per group) of culture with DHA (50µM), MEHP (20µM), DHA/MEHP (50µM and 20µM, respectively) or medium only (control). Histograms represent the mean ± SEM for each condition.

Figure 7. Testes of mouse at 13.5 dpc were cultured with DHA (50µM), MEHP (20µM), DHA/MEHP (50µM and 20µM, respectively) or medium only (control) for 3 days and then mRNAs were isolated from whole gonad. Transcriptional level of *Vasa*, *Sox9*, *Fgf9*, *Dmnt3l*, *Srebp1c*, *Pparα*, *Pparγ*, *Lxrα*, *Star*, *3β-hsd* and *Cyp11a1* was analyzed by real-time qPCR and normalized to *actin β*. Histograms represent the mean ± SEM of 5 samples (pools of whole testis - n = 4 to 5 per group) from different fetuses for each condition.

Figure 8. Testes of mouse at 13.5 dpc were cultured with DHA (50µM), MEHP (20µM), DHA/MEHP (50µM and 20µM, respectively) or medium only (control) for 24h and then mRNAs were isolated from sorted cells. Transcriptional level of *Vasa*, *Sox9*, *Fgf9*, *Pparα*, *Lxrα* and *Srebp1c* was analyzed by real-time qPCR and normalized to *actin β*. Histograms represent the mean of 1 to 4 samples (pools of whole testis - n = 3 to 6 per group before the cell sorting) from different fetuses for each condition.

Figure 1

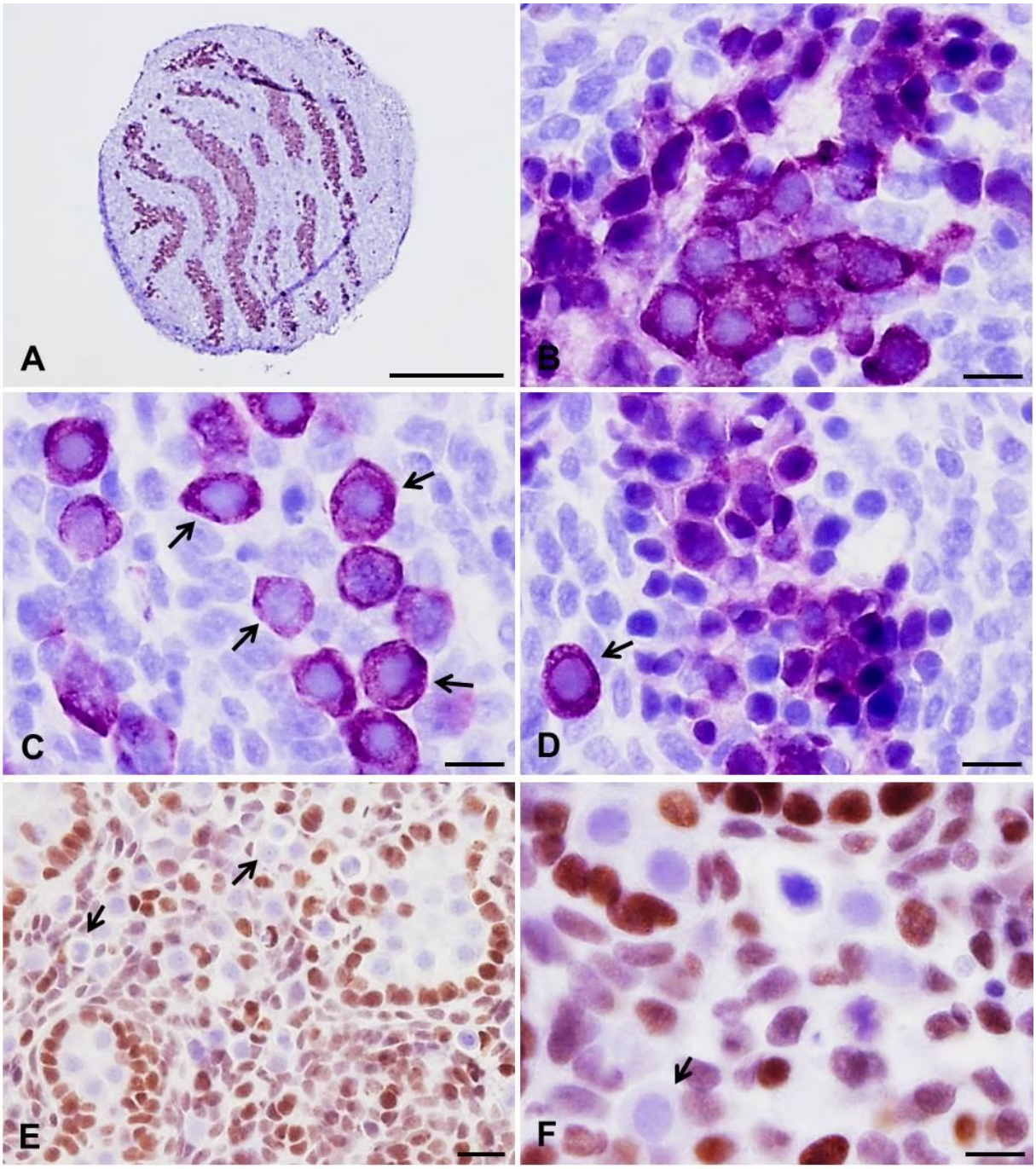


Figure 2

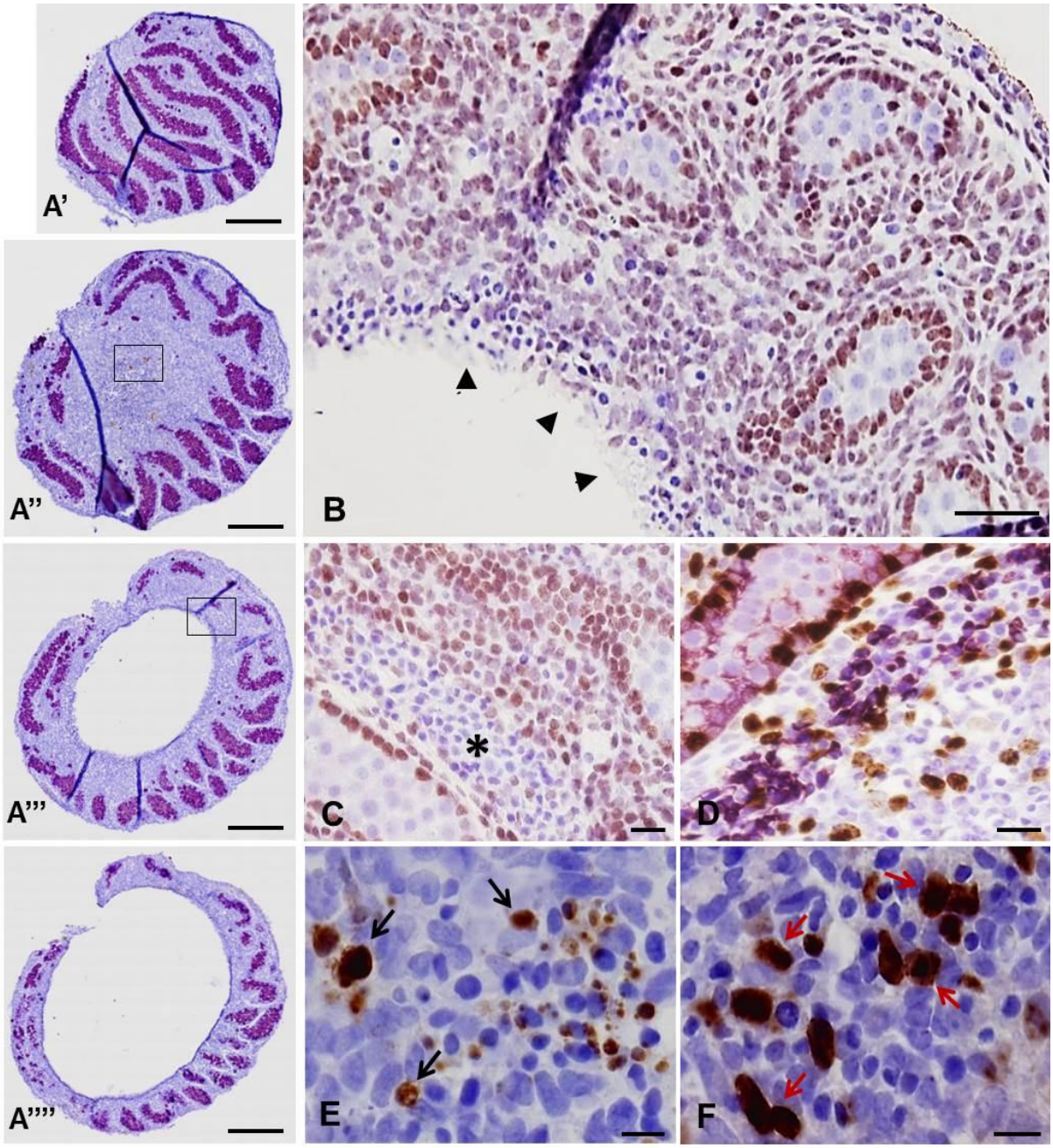


Figure 3

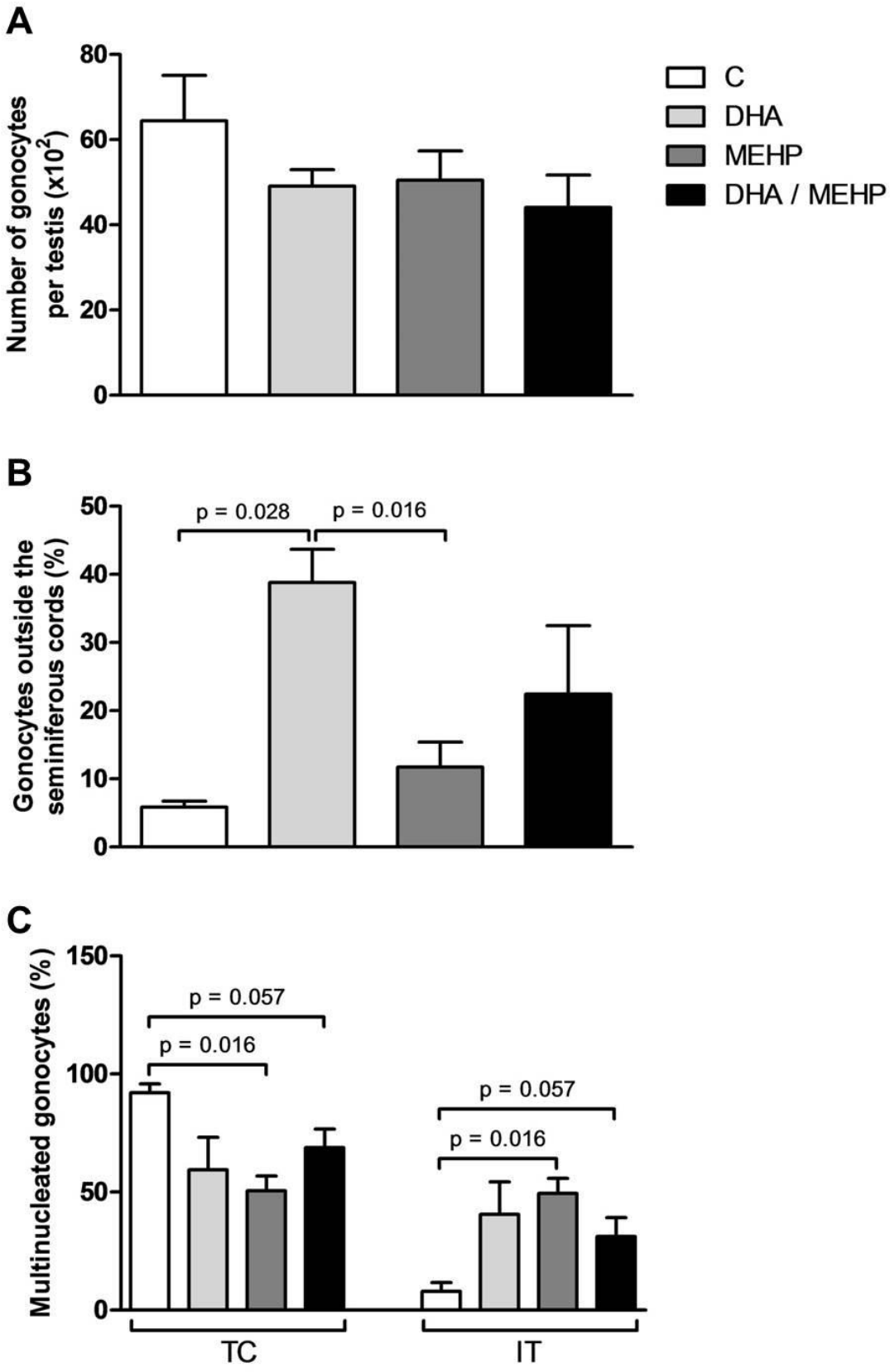

A
Number of gonocytes per testis (x10²)
80
60
40
20
0
C
DHA
MEHP
DHA / MEHP
B
Gonocytes outside the seminiferous cords (%)
50
40
30
20
10
0
p = 0.028
p = 0.016
C
Multinucleated gonocytes (%)
150
100
50
0
p = 0.057
p = 0.016
p = 0.057
p = 0.016
TC
IT

Figure 4

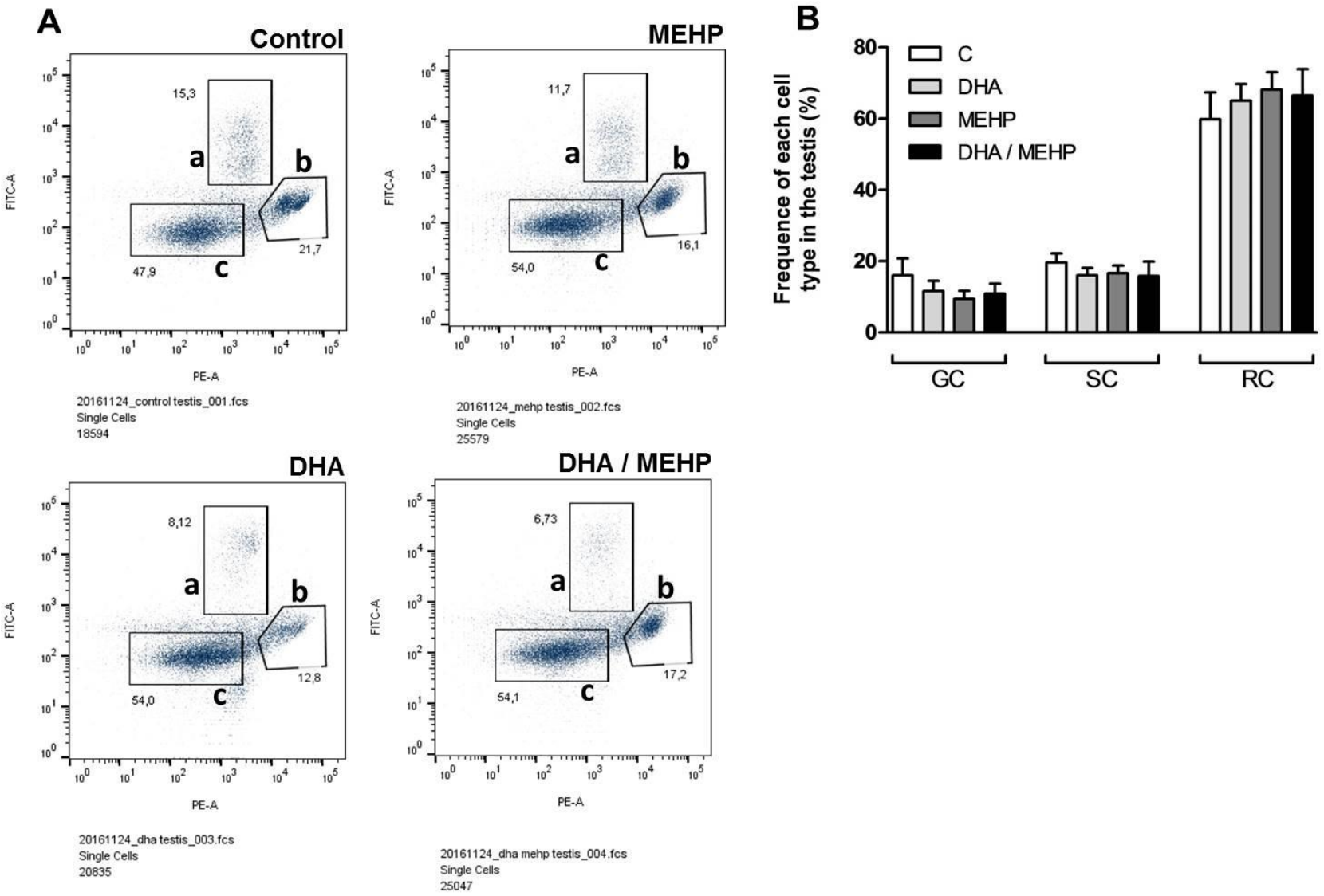

A
Control
15,3
a
b
21,7
47,9
c
FITC-A
PE-A
20161124_control testis_001.fcs
Single Cells
18594
MEHP
11,7
a
b
16,1
54,0
c
20161124_mehp testis_002.fcs
Single Cells
25579
DHA
8,12
a
b
12,8
54,0
c
20161124_dha testis_003.fcs
Single Cells
20835
DHA / MEHP
6,73
a
b
17,2
54,1
c
20161124_dha mehp testis_004.fcs
Single Cells
25047
B
Frequence of each cell type in the testis (%)
C
DHA
MEHP
DHA / MEHP
80
60
40
20
0
GC
SC
RC

Figure 5

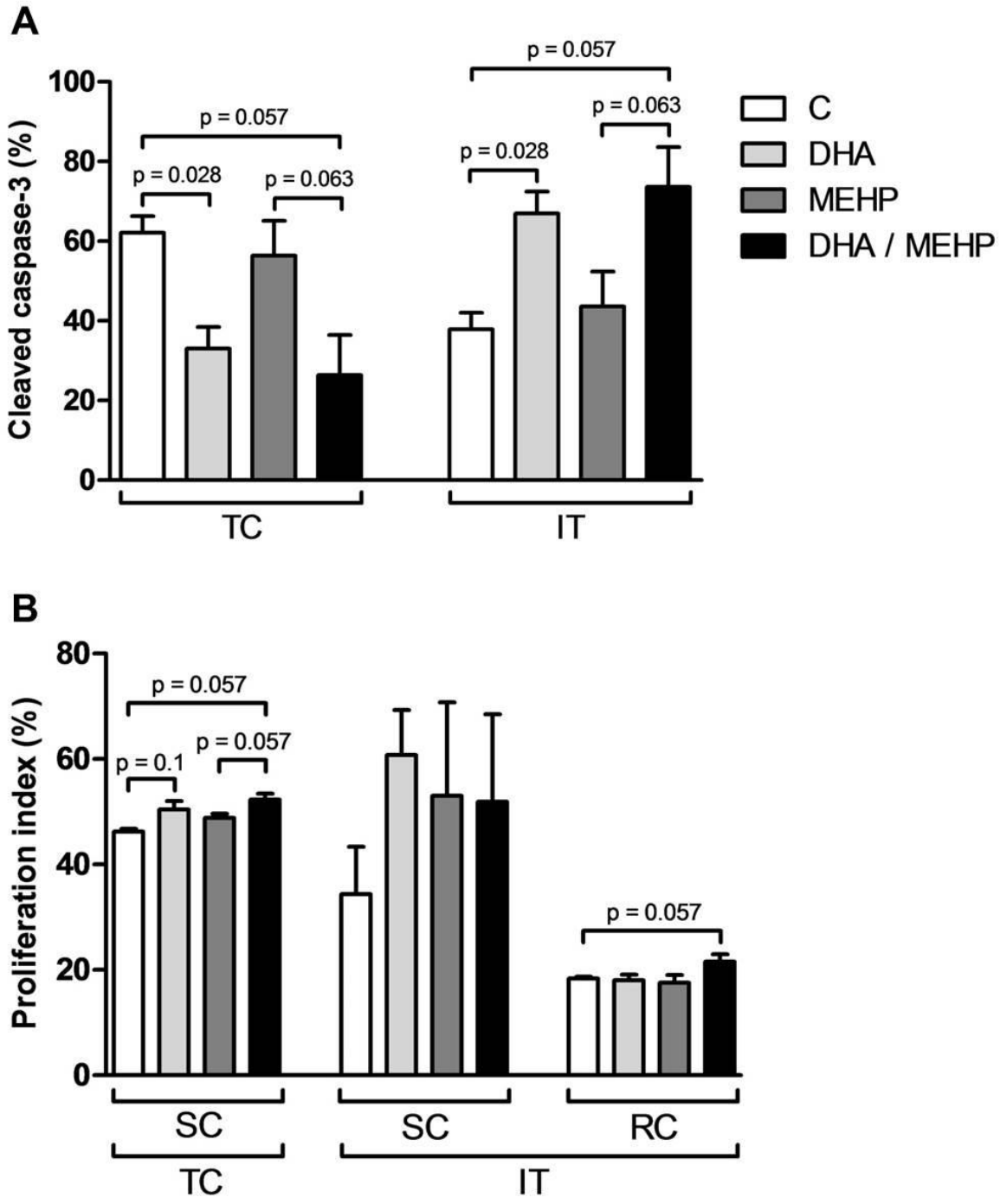

A
Cleaved caspase-3 (%)
100
80
60
40
20
0
p = 0.057
p = 0.028
p = 0.063
p = 0.057
p = 0.028
p = 0.063
TC
IT
C
DHA
MEHP
DHA / MEHP
B
Proliferation index (%)
80
60
40
20
0
p = 0.057
p = 0.1
p = 0.057
p = 0.057
SC
SC
RC
TC
IT

Figure 6

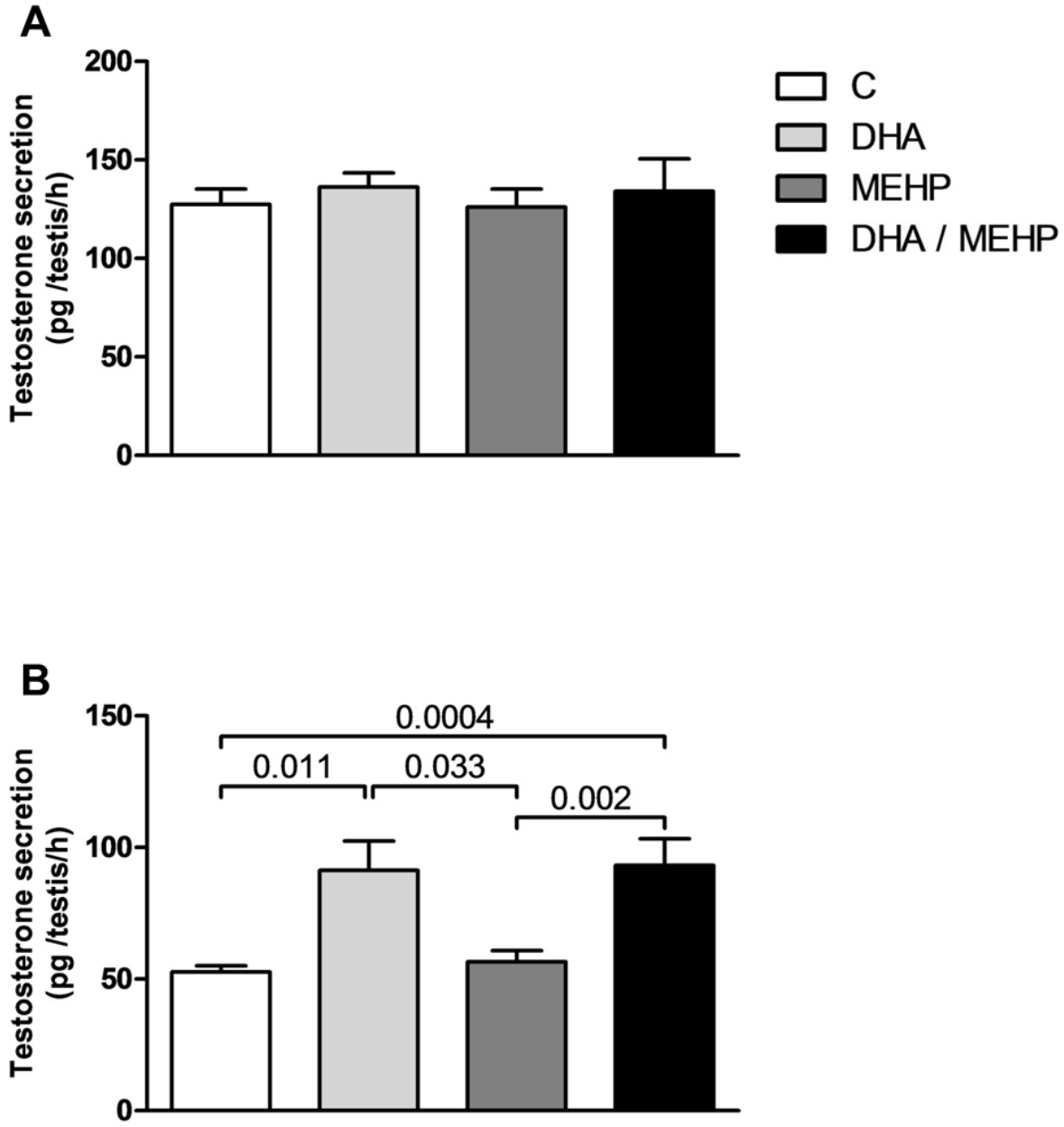

A
Testosterone secretion (pg /testis/h)
200
150
100
50
0
C
DHA
MEHP
DHA / MEHP
B
Testosterone secretion (pg /testis/h)
150
100
50
0
0.0004
0.011
0.033
0.002

Figure 7

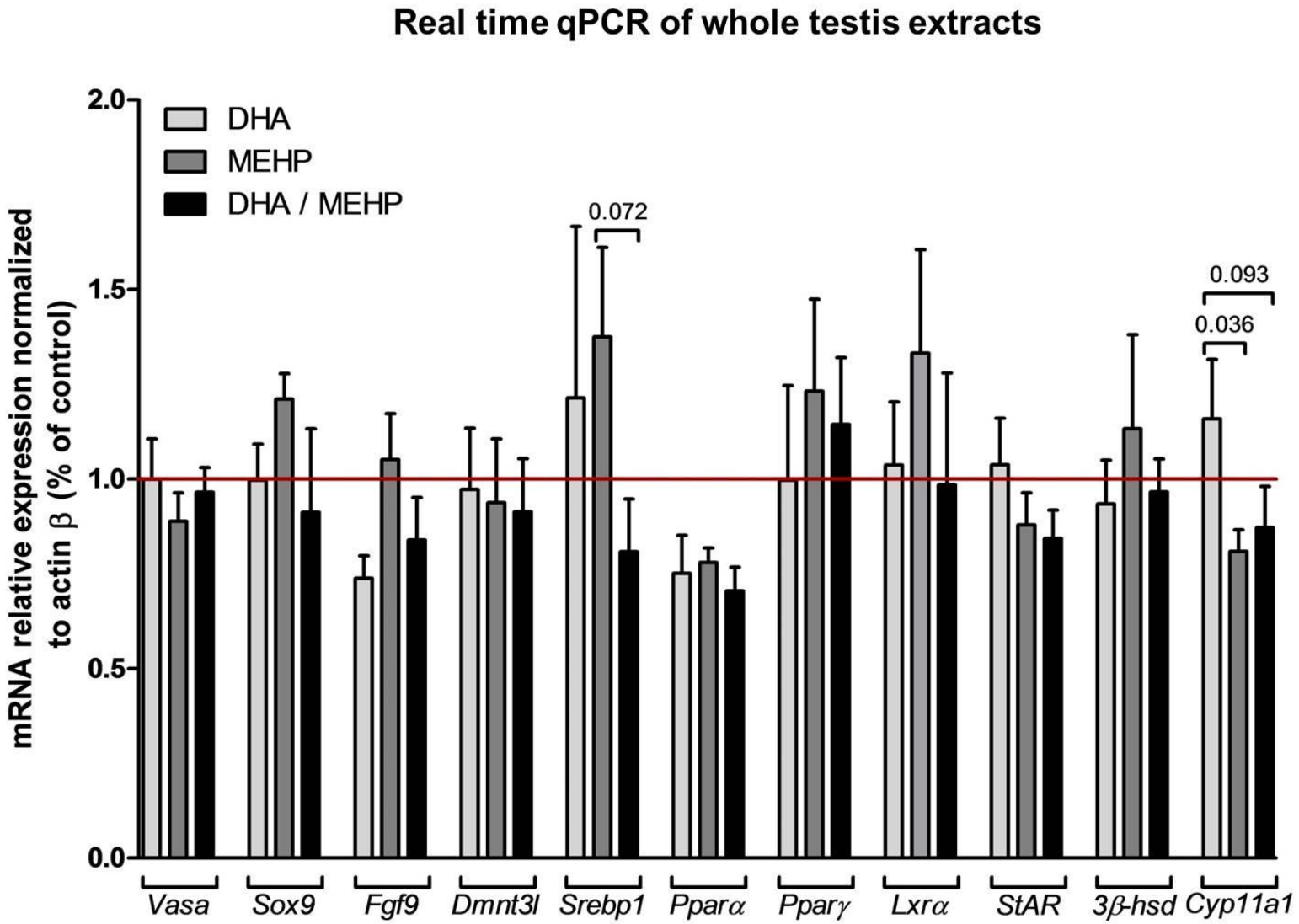
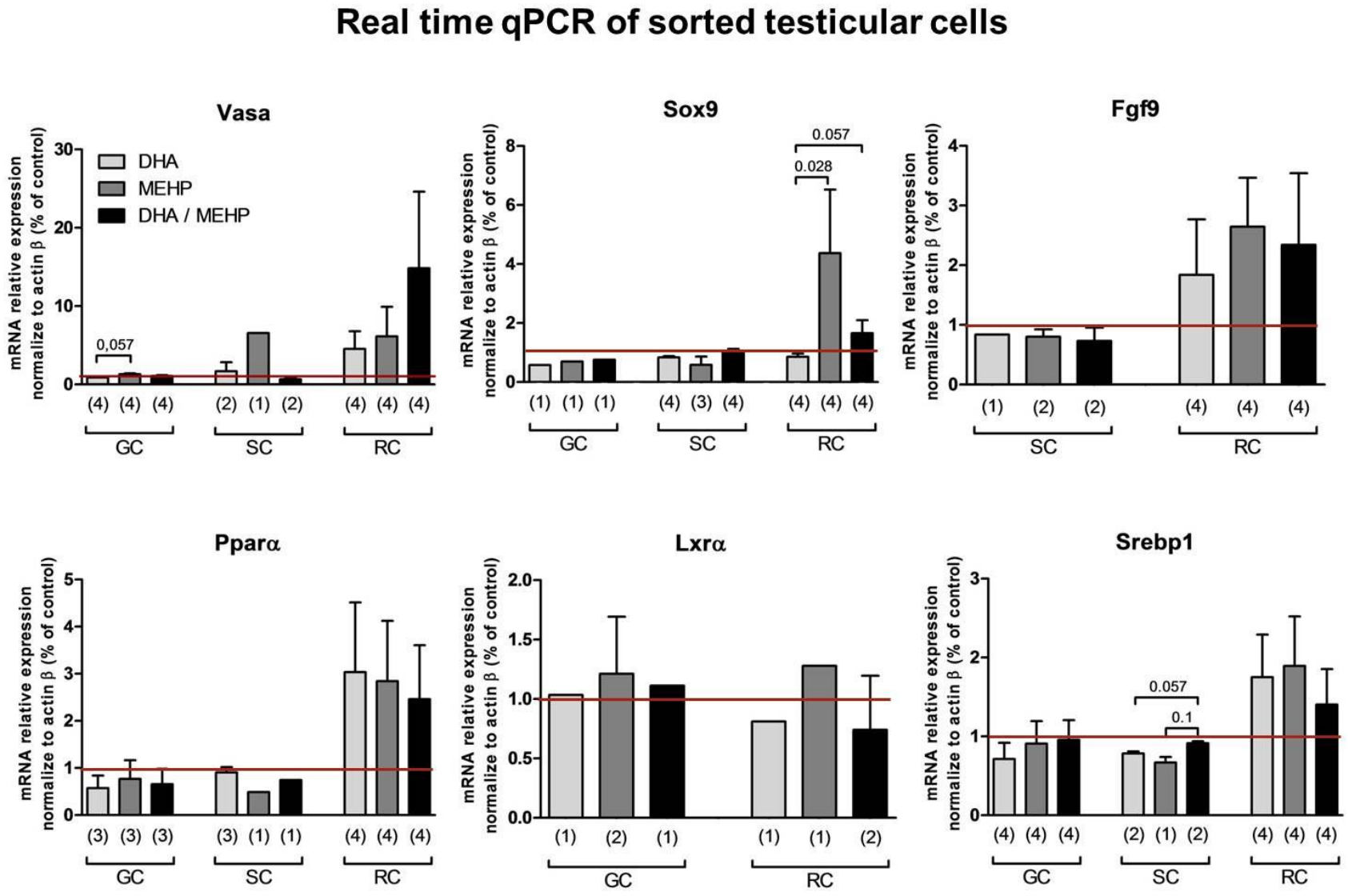

Real time qPCR of whole testis extracts
DHA
MEHP
DHA / MEHP
mRNA relative expression normalized to actin β (% of control)
2.0
1.5
1.0
0.5
0.0
0.072
0.093
0.036
Vasa
Sox9
Fgf9
Dmnt3l
Srebp1
Pparα
Pparγ
Lxrα
StAR
3β-hsd
Cyp11a1

Figure 8


Real time qPCR of sorted testicular cells
Vasa
Sox9
Fgf9
Pparα
Lxrα
Srebp1
mRNA relative expression normalize to actin β (% of control)
DHA
MEHP
DHA / MEHP
0,057
0.057
0.028
0.1
(4) (4) (4) (2) (1) (2) (4) (4) (4)
(1) (1) (1) (4) (3) (4) (4) (4) (4)
(1) (2) (2) (4) (4) (4)
(3) (3) (3) (3) (1) (1) (4) (4) (4)
(1) (2) (1) (1) (1) (2)
(4) (4) (4) (2) (1) (2) (4) (4) (4)
GC
SC
RC

V. CONCLUSÕES

A exposição pré-natal do gerbilo da Mongólia ao di-n-butil ftalato não alterou a distância anogenital ou o número de células germinativas ao nascimento. Entretanto, o DBP aumentou os níveis de testosterona ao final da primeira semana de vida, provavelmente por vias independentes dos receptores de andrógeno, e prejudicou o número de gonócitos também aos 7 dias. Além dos efeitos do ftalato, um forte efeito estrogênico foi observado, ao final da quarta semana de vida pós-natal, após exposição apenas ao óleo mineral.

A utilização do sistema de cultura organotípica revelou que o DHA induziu um extravasamento marcante de gonócitos para o tecido intersticial, um efeito pró-apoptótico sob as populações celulares do estroma, além de alterações degenerativas nas células de Sertoli do testículo fetal de camundongo. Estas últimas foram intensificadas quando em combinação com o MEHP. O DHA também estimulou a secreção de testosterona pela gônada, independentemente da incubação com o ftalato, após 72 horas de cultura.

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VII. ANEXOS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que o projeto/disciplina de pesquisa intitulado **"Impacto da exposição gestacional ao di-n-butil ftalato sobre o desenvolvimento testicular do gerbilo da Mongólia"** (protocolo nº. 089/2013 - CEUA), sob responsabilidade da Professora Doutora Rejane Maira Góes, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais deste Instituto, em reunião de 04 de dezembro de 2013.

CERTIFICATE

Certify that the project / research discipline entitled " Impact of gestational age at di-n-butyl phthalate exposure on testicular development of the Mongolian gerbil" (protocol no. 089/2013 - CEUA), under the responsibility of Professora Rejane Maira Góes, is in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Use of this Institute, at the meeting of December 04, 2013.

São José do Rio Preto, 04 de dezembro de 2013.

Prof. Dr. Luiz Henrique Florindo
Vice-Presidente da CEUA

Instituto de Biociências, Letras e Ciências Exatas – Comissão de Ética no Uso de Animais
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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em resvistas científicas ou anais de congresso sujeitos a arbitragem, que constam de minha Dissertação/Tese de Mestrado/Doutorado intitulada **"ESTUDOS EXPERIMENTAIS DO EFEITO DO DI-N-BUTIL FTALATO E DO MONO-(2-ETILHEXIL) FTALATO SOBRE O DESENVOLVIMENTO TESTICULAR DE ROEDORES"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 20 de julho de 2017

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